PRIMING AND TARGET RECOGNITION OF AUTOIMMUNE PATHOGENIC T CELLS

DISSERTATION

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DISCLAIMER

The thesis is based on the following publications:

- ***Induction of primary T cell immunity in the absence of secondary lymphoid tissues***

  Melanie Greter, Burkhard Becher

  (submitted)

- ***Antigen presentation in autoimmunity and CNS-inflammation: How T lymphocytes recognize the brain***

  Burkhard Becher, Ingo Bechmann, Melanie Greter

  Journal of Molecular Medicine 2006 (1).

- ***Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis***

  Melanie Greter, Frank L Heppner, Maria P Lemos, Berhard M Odermatt, Norbert Goebels, Terri Laufer, Randolph J Noelle, Burkhard Becher

  Nature Medicine 2005 (2)

- ***Experimental autoimmune encephalomyelitis repressed by microglial paralysis***

  Frank L Heppner, Melanie Greter, Denis Marino, Jeppe Falsig, Gennadij Raivich, Nadine Hövelmeyer, Ari Waisman, Thomas Rülicke, Marco Prinz, Josef Priller, Burkhard Becher, Adriano Aguzzi

  Nature Medicine 2005 (3)

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ABBREVIATIONS

Ab:	Antibody
Ag:	Antigen
APC:	Antigen-presenting cell
BM	Bone marrow
CNS:	Central nervous system
CTL:	Cytotoxic T lymphocyte
DC:	Dendritic cell
EAE:	Experimental autoimmune encephalomyelitis
FDC:	Follicular dendritic cell
GC:	Germinal center
Ig:	Immunoglobulin
IFNγ:	Interferon gamma
IL:	Interleukin
LPS:	Lipopolysaccharide
LTα:	Lymphotoxin alpha
LTβR:	Lymphotoxin beta receptor
MBP:	Myelin basic protein
MHCI:	Major histocompatibility complex class I
MHCII:	Major histocompatibility complex class II
MOG:	Myelin oligodendrocyte glycoprotein
MS:	Multiple sclerosis
NFκB:	Nuclear factor kappa B
NIK:	NFκB-inducing kinase
NK:	Natural killer cell
NKT:	Natural killer T cell
PLP:	Proteolipid protein
RAG:	Recombination-activation gene
s.c.:	Subcutaneous
SLT:	Secondary lymphoid tissue
TCR:	T-cell receptor
TGF-β:	Transforming growth factor-beta
T_H:	T-helper cells
Tc:	Cytotoxic T cell
TMEV-IDD:	Theiler's murine encephalomyelitis virus-induced demyelinating disease
TNF-α:	Tumor necrosis factor alpha
TLR:	Toll-like receptor
Treg:	Regulatory T cell
Wt:	Wild-type

SUMMARY

Subcutaneuos immunization with protein antigen (Ag) leads to the licensing of local dendritic cells (DCs) and the initiation of T cell priming. At the site of immunization, DCs take up the antigen (Ag) and subsequently migrate into the regional lymph nodes where they initiate the activation and expansion of T cells recognizing their cognate Ag. The structural environment within secondary lymphoid tissues (SLTs) is considered essential for the initiation of adaptive immunity. However, we discovered that immunization-induced T cell priming can occur outside of such dedicated SLTs. While B cell priming is highly dependant on the topography of SLTs, effective T cell immunity can occur completely independent of lymphoreticular structures. In the absence of SLTs, the liver displays the potential to serve as an alternative site for T-cell priming.

After successful T cell priming occurred, T cells migrate through the body's tissues in search of their cognate Ag, which needs to be presented by an Ag-presenting cell (APC) in the context of major histocompatibility complex class II. When a self-Ag, such as CNS-myelin is the prime target, T cells re-encouter their cognate Ag presented by central nervous system (CNS)-associated DCs in perivascular spaces, which is an absolute requirement for the development of experimental autoimmune encephalomyelitis. After this second encounter with their cognate Ag, myelin-reactive T cells cross the glia limitans and enter the CNS parenchyma where they interact with CNS-resident microglia. Microglia do not serve as classical APCs, however they will produce a host of vasoactive substances, cytokines and chemokines leading to the invasion of other leukocytes and the development of a tissue lesion. While the precise molecular mechanisms of the disease pathogenesis will need to be elucidated in the future, our findings have provided a great leap of our understanding of the spatial and temporal process occurring during autoimmune diseases.

ZUSAMMENFASSUNG

Subkutane Immunisierung mit Protein-Antigen führt zur Aktivierung von dendritischen Zellen (DCs) und T-Zell „*priming*“. Nachdem DCs das Antigen (Ag) lokal aufgenommen haben, migrieren sie in die regionalen Lymphknoten, wo sie die Aktivierung und Expansion von T Zellen induzieren, welche das Ag erkennen. Sekundäre lymphatische Organe (SLOs) mit ihren klar abgegrenzten T- und B-Zellzonen werden als auschlaggebend für die Entwicklung adaptiver Immunität betrachtet. Wir haben jedoch entdeckt, dass Immunisierungs-induziertes T-Zell „*priming*“ auch ausserhalb von solch definierten Strukturen stattfinden kann. Während effektive B-Zellaktivierung von der Topographie in SLOs abhängig ist, funktioniert T-Zellaktivierung ohne lymphoretikuläre Strukturen. Wir haben herausgefunden, dass in der Abwesenheit von SLOs möglicherweise die Leber als Organ für T-Zell *„priming“* dienen könnte.

Nachdem T-Zell „*priming*“ erfolgreich stattgefunden hat, verlassen die T Zellen die SLOs um den Körper nach ihrem spezifischen Ag zu durchsuchen, das auf dem „major histocompatibility complex class II“ von Ag-präsentierenden Zellen (APZs) präsentiert weren muss. Wenn ein Selbst-Ag das Angriffsziel ist, wie z.B Myelin im Zentralnervensystem (ZNS), treffen die Myelin-reaktiven T Zellen im perivaskulären Raum auf DCs, welche ihnen ihr Ag präsentieren. Dieser Schritt ist absolut entscheidend für die Entstehung der experimentellen Autoimmun-Enzephalomeylitis. Nach der Wiedererkennung des Ags migrieren die Myelin-reaktiven T Zellen durch die *Glia Limitans* und dringen in das Parenchym des ZNS ein. Dort interagieren sie mit ZNS-angesiedelten Microgliazellen, die daraufhin aktiviert werden. Microglia dienen nicht als klassische APZs, sekretieren jedoch vasoaktive Substanzen, Zytokine und Chemokine, wodurch die Invasion von Leukozyten und die Entwicklung einer Läsion herbeiführt wird. Während die genauen molekularen Mechanismen des Krankheitsverlaufes zukünftig noch genauer untersucht werden müssen, haben unsere Daten einen grossen Erkenntnisgewinn im räumlichen sowie zeitlichen Ablauf autoimmuner Erkrankungen erbracht .

INTRODUCTION

ADAPTIVE IMMUNITY AND AUTOIMMUNITY

The mammalian immune system constitutes antigen (Ag)-non-specific innate immunity and Ag-specific adaptive immunity. Innate immunity comprises the first line of defense against pathogens whereby phagocytic cells (e.g. macrophages, neutrophils) and natural killer (NK) cells are considered key players in eliminating infections. A prime feature of innate immunity is its capacity to recognize a vast array of patterns shared by many pathogens through germline-encoded pattern recognition receptors (Toll-like receptors (TLRs)). While innate immune responses can act rapidly immediately after recognition of molecular patterns, adaptive immunity requires significantly more time to develop. These two arms of immunity are not separate entities but heavily intertwined and influence one another. Adaptive immunity is defined by Ag-specificity, diversity and immunological memory. While these features allow highly complex and specific immune recognition, adaptive immunity can also function improperly leading to the failure of self-non-self recognition and the development of allergies, asthma or autoimmune diseases such as multiple sclerosis (MS).

Adaptive immune responses can be divided into cellular and humoral immunity. The humoral branch of the immune system comprises B cells which differentiate into antibody (Ab)-secreting plasma cells whereas the cellular immune response is characterized by T cells recognizing Ag presented on major histocompatibility complex (MHC) by antigen presenting cells (APCs). $CD4^+$ T cells recognize Ags that are associated with MHC class II (MHCII) molecules, whereas $CD8^+$ T cells bind Ag-MHC class I (MHCI) complexes. “Professional” APCs such as dendritic cells (DCs) display the unique hallmark to initiate primary immune responses by Ag-presentation to naïve T cells (4,5). On the other hand, “non-professional” APCs like B cells are engaged in secondary T cell responses by Ag-presentation to primed T cells (6).

In this thesis I will discuss the role and function of secondary lymphoid tissues (SLTs) in the priming and activation of T lymphocytes, investigate how such T cells, when their cognate Ag is self-Ag, recognize their target in the central nervous system (CNS) and delineate the participation and function of microglia in regulating the events leading to the unleashing of an inflammatory cascade.

EVOLUTION OF ADAPTIVE IMMUNITY AND THE ROLE OF PERIPHERAL LYMPHOID TISSUES

EVOLUTION OF ADAPTIVE IMMUNITY

In contrast to innate immunity, which exists in all vertebrates, invertebrates and to some extent in plants, adaptive immunity is only found in vertebrates and is characterized by lymphocytes, Ag-receptors (Abs and T-cell receptors (TCRs)), MHCI, MHCII and immunological memory (18). Adaptive immunity has evolved in the oldest jawed vertebrates (Gnathostomes such as cartilaginous fish including sharks) with the emergence of the unique recombination-activating gene (RAG), which rearranges Ag-binding receptors by somatic recombination. RAG is held to be the key feature of adaptive immunity as it creates a vast diversity of (non-germline generated) T and B cell receptors highly specific for limitless recognition of any biological molecule (19).

Most of the jawless vertebrates (Agnathans) are extinct except for the lampreys and the hagfish. It has recently been reported that lampreys, which lack all lymphoid tissues, display adaptive immune responses executed with RAG-independent mechanisms. This new type of variable lymphocyte receptors (VLRs) is thought to be an ancestral prototype for adaptive immunity (20). It is assumed that with the evolution of a jaw in relation to a predatory life adaptive immunity was brought about. The jaw hypothesis states that adaptive immunity arose in the gastrointestinal region of primitive jawed fish (Placoderm) as a result of enhanced infections due the altered feeding behavior (21).

After the emergence of T and B lymphocytes in jawed fish, the evolution of adaptive immunity has invented new specialized organs in higher vertebrates: Secondary lymphoid tissues (SLTs) such as lymph nodes (LNs). The micro-environment of SLTs

with the distinct T and B cell areas is considered essential for the sequestration of Ag and the initiation of T and B cell-mediated immunity. Interestingly, immunoglobulin (Ig)-class switching and germinal center (GC) formation, the site where B-cell differentiation into plasma and memory cells occurs, are lacking in cartilaginous fish and only appeared at the time of the divergence of amphibians (18). This observation would imply that cell-mediated immunity evolved earlier than "modern" humoral immune responses and raises the question whether productive T and B cell immunity requires the same structural environments.

SECONDARY LYMPHOID TISSUES

SLTs include LNs, the spleen, Peyer's patches, gut- and bronchus-associated lymphoid tissues, and are considered essential for the development of adaptive immune responses. SLTs are greatly organized structures and have evolved in order to trap and concentrate Ag and to allow the interaction of Ag-laden APCs with rare Ag-specific T and B cells. Prevailing immunological dogma dictates that only if the Ag reaches or is transported by APCs to SLTs an immune response is generated otherwise it is ignored by lymphocytes (22,23,24,25). The highly organized SLTs contain defined compartments consisting of T cell areas (partacortex in LNs and periarterial lymphoid sheath in the spleen) and B cell areas (cortex containing GCs) (Figure 1 and 2).

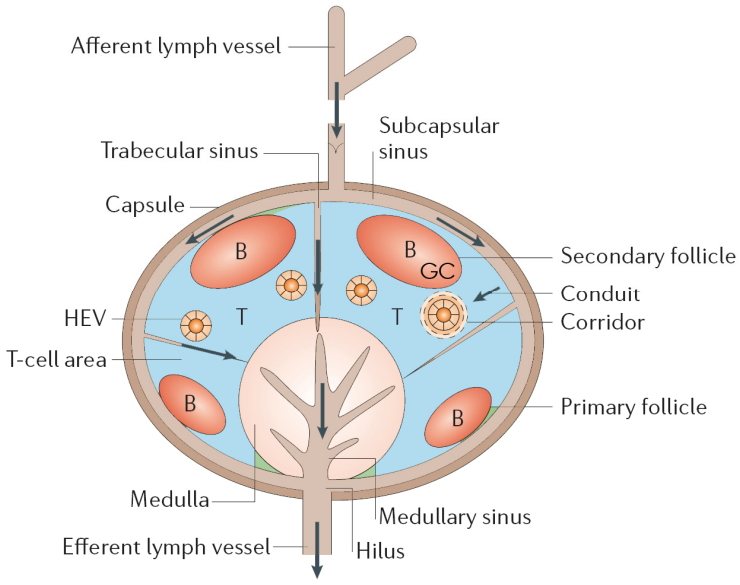

Figure 1: Basic structure of a SLT. Modified and adapted from Aloisi *et al*. (26).

The paracortex is the site where circulating T lymphocytes enter the LN and interact with Ag-laden DCs. T lymphocytes specific for the foreign Ag are thus activated and clonally expand. Upon differentiation, T cells either leave the LNs in order to migrate through the body's tissues in search of their cognate Ag displayed by resident APCs or they home to the B cell areas to assist in Ag-specific Ab production. After receiving T cell help, which initially takes place in the paracortex, activated B cells migrate together with T_H cells into the cortex in order to initiate the GC reaction (27). In GCs, a network of follicular dendritic cells (FDCs) displays Ag-Ab complexes to B cells. FDCs are reticular fibroblasts (non-lymphocytic cells) specialized to promote B cell survival and proliferation. The interaction of B cells with FDCs and T_H cells is a prerequisite for their differentiation into plasma cells and the production of high-affinity Abs. In contrast to the cortex, T cell areas are structurally ill-defined. While intravital confocal microscopy has provided compelling evidence for the capacity of SLTs to host T cell priming (28,29), definitive data supporting their absolute requirement for functional T cell priming is lacking.

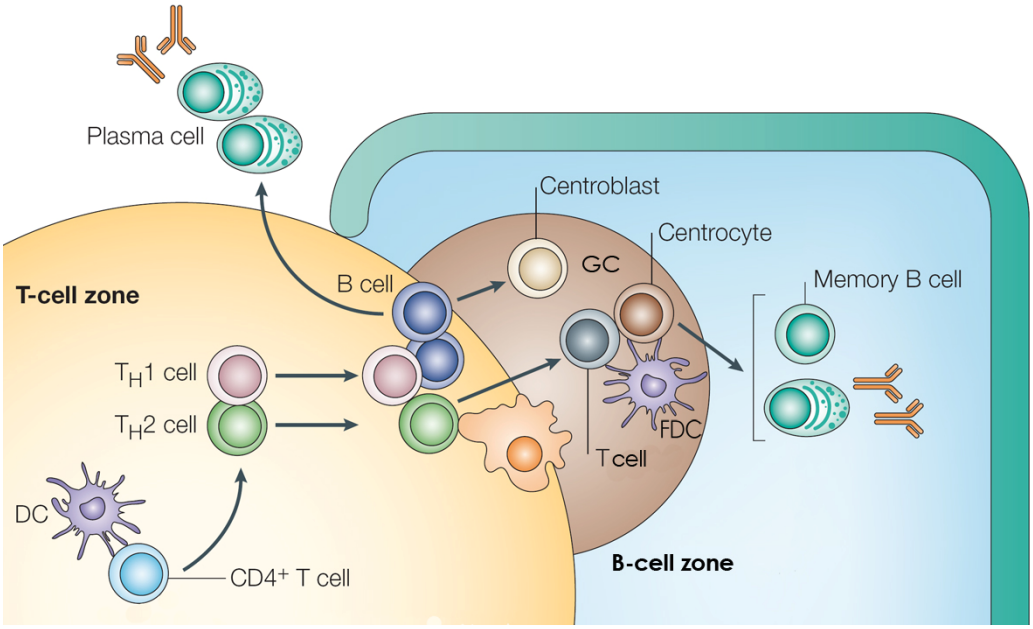

Figure 2: Pathway of B cell differentiation. Adapted and modified from Mackay *et al.* (27).

TERTIARY LYMPHOID TISSUES

Ectopic lymphoid neogenesis can develop upon chronic inflammation such as an autoimmune response or infection. The micro-architecture of these tertiary lymphoid structures (TLTs) resembles SLTs as they contain defined T cell and B cell compartments. TLTs have been described in humans e.g. for myasthenia gravis in the thymus, chronic hepatitis C in the liver or MS in the CNS (26). Such lymphoid-like structures found in MS patients were located in the meninges and comprise B cells, T cells and FDCs. It is thought that for the formation of both SLTs and TLTs the same signaling components are required. These include mainly signaling through lymphotoxin β-receptor (LTβR). In mice, transgenic expression of LTα in the pancreas induced the chronic infiltration of lymphocytes and the development of TLTs (30). However, ectopic neogenesis might depend on additional signaling pathways other than LTβR signaling. This is shown by the fact that splenectomized $LT\alpha^{-/-}$ mice, which lack all SLTs, were able to induce lymphoid neogenesis (31). For the recruitment of B cells, T cells and DCs, the expression of the chemokines CCL21, CCL19 and CXCL13 is further implicated to play a critical role (26). The underlying cause for the formation of TLTs remains speculative. In chronic inflammations the development of TLTs might exacerbate the disease. However upon infections with pathogens, TLTs could provide a benefit for the host and might even replace conventional SLTs.

ALYMPHOPLASIA MICE

The role of NFkB-inducing kinase

The nuclear factor κ B (NFκB) family of transcription factors induces the expression of a vast variety of genes involved in innate and adaptive immune responses, activation, development, cell growth and apoptosis (32). The activation of NFkB is tightly regulated and its constitutive expression has been associated with inflammatory diseases such as rheumatic arthritis and MS. In mammals, 5 different members exist which can form homo and heterodimers: NFkB1 (p50 and its precursor p105), NFkB2 (p52 and its precursor p100), RelA (p65), RelB and c-Rel. The prominent activated form comprises the heterodimers p65 with p50 or p52. They are

retained in an inactive form in the cytoplasm by inhibitory IkB proteins which mask their nuclear localization signal (NLS) (33).

Two NFkB pathways exist: The canonical pathway includes NFkB1 and RelA, and the non-canonical pathway involves NFkB2 and RelB (Figure 3). The classical pathway is activated by inflammatory stimuli (such as TNF-α and lipopolysaccharide (LPS)) through TNF receptors (TNFR), TLRs, and IL-1 receptors leading to the activation of IkB-Kinases (IKKs). The IKK complex consists of IKK1, IKK2 and IKK3 and upon activation, phosphorylates IkB proteins leading to their proteosomal degradation and thus activation and translocation of NFkB1/Rel-A into the nucleus. Members of the non-canonical pathway include CD40, LTβR, receptor activator of NF-kB (RANK) and B-cell activating factor receptor (BAFFR). This pathway is dependent on IKK1, but independent of IKK2 and IKK3. The crucial component is the NFkB inducing kinase (NIK) which activates IKK1 that in turn phosphorylates and processes p100, releasing the active form of NFkB2/Rel-B (p52-RelB) to translocate into the nucleus and activate gene transcription.

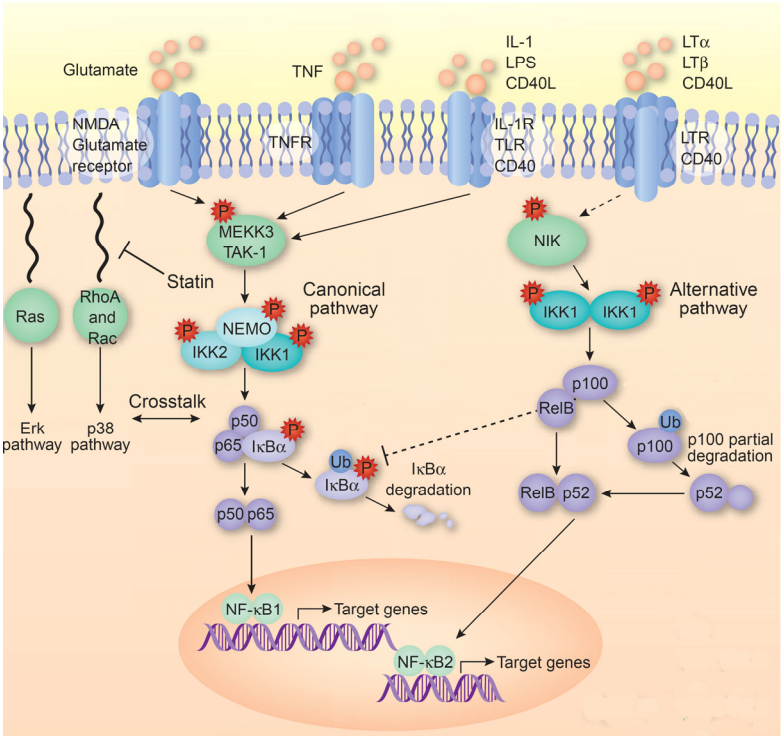

Figure 3: NFκB signaling pathways. Adapted and modified from Youssef *et al.* (34).

Alymphoplasia mice

Alymphoplasia (*aly/aly*) mice are characterized by a complete lack of LNs and Peyer's patches due to a point mutation in NIK (35,36,37,38). The spleen of *aly/aly* mice is devoid of well-defined lymphoid structures and the thymus does not display discrete borders between the cortex and the medulla. LTβR$^{-/-}$ and NIK$^{-/-}$ mice exhibit similar developmental defects as *aly/aly* mice (38,39). In addition to the lymphoid malformations caused by the disrupted LTβR-signaling, *aly/aly* mice are also immuno-deficient. This became evident by the fact that the development of GCs, isotype-switching and affinity maturation is entirely abolished. The serum levels of IgG and IgA in a*ly/aly* mice are virtually absent while the amount of IgM is one third in comparison to control mice (24). In splenectomized *aly/aly* mice the Ab response is completely ablated (40).

Aly/aly mice are not able to reject allogeneic skin grafts (24,41) while splenectomized *aly/aly* mice accept vascularized organ transplants indefinitely suggesting that allograft responses to vascularized organs are not initiated in the graft itself but rather in SLTs. Moreover rejection of tumors in *aly/aly* mice was abrogated, which was attributed to the fact that tumor cells cannot be trapped in LNs in order to induce priming of CTLs (42).

Aly/aly mice are highly susceptible to viral infections such as vesicular stomatitis virus (VSV) due the absent Ab switch to the protective IgG. Injections with lymphocyte choriomeningitis virus (LCMV) and vaccinia virus (VV) resulted in delayed elimination or life-long persistence of the virus respectively (43). The structural malformations were considered the underlying cause for this inefficient T and B cell cooperation. This notion was further supported by using BM-chimeric mice, which revealed that the weak CTL-response to LCMV in *aly/aly* mice is caused by the absence of LNs and not from an intrinsic T cell defect. However, for B cells an intrinsic defect independent of SLTs was found (44). Garceau *et al*. have shown that NIK is essential for T cell dependant B cell maturation through CD40 but that NIK signaling upon CD40 engagement is dispensable for the activation of DCs (37).

Recently it has been discovered that NIK itself is vital for T cell activation in general and in the context of autoimmunity. NIK-deficient T cells displayed reduced proliferation and IL-2 production in response to TCR cross-linking (45). Sprent and

coworkers explained these T cell defects in *aly/aly* mice (46) with the action of a new identified subpopulation of memory $CD4^+$ T cells ($CD25^-$ $foxp3^-$) which mediated immuno-suppression. When this inhibitory population was removed, naïve *aly/aly* T cells showed hyper-responsive proliferation to T cell receptor (TCR)-CD28 ligation. Adoptive transfer of such hyper-responsive naïve *aly/aly* T cells (depleted of suppressive T cells) into recipient mice ($RAG^{-/-}$) caused the development of autoimmune disease. The hyper-responsiveness was attributed to reduced p100 synthesis leading to unregulated nuclear translocation of NFkB1. Therefore the NFkB2 signaling pathway including NIK might acts as a regulatory brake for the NFkB1 pathway.

DENDRITIC CELLS: PROFESSIONAL APCS AND REGULATORS OF IMMUNE RESPONSES

DCs are the most powerful APCs in higher vertebrates and conduct major control over the development of an immune response. The immunological outcome of immunity is mainly influenced by their activation state. Upon exposure to microbial products, DCs induce immunity whereas in the steady state, they are critical for the maintenance of tolerance.

As the "sentinels" of the immune system, DCs reside as immature cells in peripheral tissues in constant search of pathogens. Ags are captured by DCs through receptor-mediated endocytosis via C-type lectins (e.g. DEC-205), Fcγ receptors or non-specifically through macro-pinocytosis or phagocytosis. Internalized Ag is processed in acidic endocytic compartments by proteases and subsequently displayed in association with MHCII molecules on the surface of the cell (7,8). Ag capture and maturation stimuli (inflammatory mediators and microbial products) induce the differentiation from the immature Ag-capturing cell to a mature APC. Mature DCs downregulate their endocytic capacities, upregulate Ag-MHCII complexes and costimulatory molecules (e.g. CD40, CD80 and CD86). The expression pattern of chemokine receptors necessary for migration is subsequently changed. CCR7 enables DCs to migrate into the T cell zone of SLTs (9), CCR5 is important for the recruitment of DCs into inflammatory sites (10) and CCR6 plays a role for migration

to epithelial cell surfaces (11). For the initiation of T cell responses, the migration of DCs into SLTs is thought to be obligatory. There, DCs present Ag to T cells leading to their activation and expansion. The differentiation of naïve T cells into effector cells is dependent on the cytokines secreted by activated mature DCs. IL-12 and IL-18 promote the differentiation into $CD4^+$ T_H1 cells, on the other side the release of IL-23 mediates the polarization to $CD4^+$ T_H17 cells.

In addition to the Ag-specific priming of helper (T_H) and cytotoxic (T_C) T cells, DCs can activate innate protective cells such as NK cells or natural killer T (NKT) cells. An extensive array of pathogens is recognized by DCs through "innate" TLRs (12), which trigger their cytokine production (e.g. IL-12, IFN-α) as well as maturation necessary for the communication between innate and adaptive immunity. IL-12 secretion by DCs stimulates NK cells to release IFNγ and increases NK-cell cytotoxicity (13). TNF-α produced by NK cells on the other hand influences DC maturation. Thus DCs form an essential link between innate and adaptive immune responses.

TOLERANCE VERSUS AUTOIMMUNITY

Central tolerance in primary lymphoid organs during early maturation of lymphocytes leads to the deletion of self-reactive T cells (in the thymus) and B cells (in the bone marrow (BM)). This mechanism is however incomplete and cells recognizing self components can escape negative selection which may cause the development of autoimmune diseases. MS is an example of such an inappropriate cellular immune response directed against myelin components of the CNS (see page 17, "Multiple sclerosis"). This inflammatory autoimmune disease is thought to be mediated by myelin-reactive T cells, which have escaped tolerance.

In order to silence auto-reactive cells, a "back-up" mechanism in the periphery exists termed peripheral tolerance. It is widely accepted that tolerogenic DCs are the primary mediators of peripheral tolerance (14). Tolerogenic DCs resemble immature DCs whereby the common features are the low expression of MHCII, CD40 and CD80/86 due to the absence of inflammatory molecules and "danger signals" (15). Peripheral tolerance is brought about through different mechanisms. Tolerogenic DCs can induce the deletion of reactive T cells, render them unresponsive (anergy) or

initiate the differentiation into regulatory T cells (Tregs). The release of immunosuppressive cytokines such as IL-10 and TGF-β by tolerogenic DCs drives the development of IL-10 producing Tregs. Collectively, the current view is that mature DCs control immunity to Ags and immature DCs tolerize T cells to self-antigens which is essential to prevent autoimmunity.

DENDRITIC CELL SUBTYPES AND FUNCTIONS

Different subtypes of DCs exist which can be distinguished by their cell-surface markers, their various functional properties, their location and their cytokine production. In mice, the two main subtypes are designated lymphoid and myeloid DCs (16). Lymphoid DCs are characterized by CD8α expression whereas the myeloid DCs are defined by the marker CD11b. Both subpopulations express CD11c, are capable of Ag-uptake and presentation to T cells. It remains unclear whether the distinct DC subtypes derived from the same or different hematopoietic lineages (16,17) and how their functional differences might reflect the polarization of an immune response. Lymphoid DCs produce higher levels of IL-12 and skew $CD4^+$ T cells to the production of T_H1 cytokines. On the other hand, myeloid DCs prime $CD4^+$ T cells to produce T_H2 cytokines (7) and are more phagocytic than lymphoid DCs. Lymphoid DCs are often implicated in regulatory T cell responses (14). It is not clearly established whether different DC subpopulations have the capacity to induce immunity or tolerance only depending on their maturation state or whether a specialized tolerogenic DC type exists. Despite the distinct functions by various DC subsets, they exhibit considerable plasticity and redundancy in driving immune responses.

THE PATHOGENESIS OF CNS-AUTOIMMUNE DISEASES

MULTIPLE SCLEROSIS

MS is the most common autoimmune disease of the central nervous system (CNS) and 1 in 900 individuals is affected in Switzerland. MS is characterized by inflammation, demyelination and axonal loss in the white matter. Although the etiology of MS remains to be established, the most widely accepted view is that immune tolerance to self (myelin) is broken, which results in the expansion of myelin-reactive T cells and their migration into the CNS. The disease is thought to be mediated by $CD4^+$ T cells auto-reactive against components of the myelin sheath (47). This notion is supported by the histopathologic observations of the presence of activated T cells in the perivascular spaces of inflammatory lesions (sites of demyelination). These infiltrates correspond to the lesions seen with magnetic resonance imaging (48,49,50) and were detected in the periventricular white matter, brain stem, cerebellum and spinal cord white matter.

The disease is most prevalent in young adults of Caucasian heritage with female preponderance (sex ratio female:male is 1.8). MS can be classified into 3 clinical subtypes: Primary progressive, secondary progressive or relapsing-remitting while the latter is the most frequent form. Common clinical features comprise paralysis, visual impairment, sensory disturbances and fatigue. It is widely believed that genetic factors may play a critical role in the susceptibility to the disease. Certain genes linked to the human leukocyte antigen (HLA) complex were found to exert an influence on the pathogenesis of MS (HLA-DR, HLA-DQ, TNF-α). A current view is that the myelin attack could be triggered by microbes and virus (e.g. Epstein-Barr virus) which proteins share homologies to myelin proteins. This concept is termed molecular mimicry.

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Experimental autoimmune encephalomyelitis (EAE) serves as the animal model for MS and can be induced in susceptible rodent strains by subcutaneous (s.c.) immunization with myelin Ags such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) or proteolipid protein (PLP).

The fact that the disease can be transferred from one animal to another by the adoptive transfer of encephalitogenic $CD4^+$ T cells supports the notion that EAE is mainly mediated by the actions of $CD4^+$ T cells (51). The role of $CD8^+$ T cells remains controversial (52,53). While several groups could clearly demonstrate that $CD8^+$ T cells restricted to an MHCI myelin Ag can transfer EAE (54,55,56), the lack of $CD8^+$ T cells in actively immunized mice worsens EAE development (57,58) .

Studies of the EAE model have helped define the sequence of immunopathological events involved in the development of autoimmune CNS-directed inflammatory disease. This sequence can be sub-divided into two major phases: T cell priming/activation and effector phase. At the site of immunization, local DCs capture myelin Ags and subsequently migrate from non-lymphoid peripheral tissues through afferent lymphatics into the draining LNs (7,8). Microbial adjuvants administered with the vaccine (e.g. *Mycobacterium Tuberculosis* in Complete Freund's adjuvant) induce the maturation of DCs which are then licensed to initiate the activation and expansion of neuroantigen-reactive T cells within SLTs (47). In the subsequent recruitment and effector phase, activated myelin-reactive T cells migrate to the CNS where they re-encounter their cognate Ag, in the context of MHCII molecules (59,60). After a few days a substantially increased number of activated T cells enter the CNS, fostering subsequent T cell migration deeper into the CNS parenchyma (61). They can now cause tissue destruction leading to, depending on the model, demyelination and ultimately neurologic deficit. During this phase, microglial cells are thought to provide additional Ag-specific as well as MHC- independent signals for invading inflammatory T cells.

Encephalitogenicity

Upon activation by APCs, $CD4^+$ T_H cells can differentiate into distinct subsets depending on their cytokine environment. T_H1 cells are characterized by their IFNγ, IL-2 and TNF-α secretion, T_H2 cells produce IL-4, IL-5, IL-10 and IL-13. In general, T_H1 cells regulate cell-mediated immunity whereas T_H2 cells mediate humoral immune responses. One subset can promote the differentiation of its own subset while the development of the other subset is antagonized. T_H1 cells were proposed to induce organ-specific autoimmunity and have been implicated in the pathogenesis of EAE and MS (62). Conversely, T_H2 cells can suppress EAE and MS, although they may exacerbate these diseases in immuno-compromised hosts (63). Thus, the final composition of the T_H cell response to Ag determines whether the outcome of infectious, inflammatory and autoimmune responses is beneficial or detrimental.

The T_H1/ T_H2 concept has recently undergone a significant paradigm shift. This shift was brought about by the observation that the lack of the major T_H1 cytokine IFN-γ exacerbated the disease (64) and renders even resistant mouse strains EAE-susceptible (65,66). Furthermore, the most critical cytokines promoting T_H1 polarization are IL-12 and IL-18, which are both predominantly secreted by activated DCs and monocytes/macrophages. Mice lacking IL-12 ($p35^{-/-}$) were shown to be hyper-susceptible to EAE (67,68,69). IL-$18^{-/-}$ mice as well as IL-$12^{-/-}$/ IL-$18^{-/-}$ double knock-out mice develop EAE with the same disease severity and kinetics as wild-type (wt) mice (70). The discovery that $p40^{-/-}$ mice are resistant to EAE has led to the identification of a new cytokine IL-23 which promotes a novel $CD4^+$ T_H subset distinct from T_H1 and T_H2 (T_H17). IL-23 consists of the unique p19 subunit and the common p40 subunit which is shared with IL-12. IL-23 is considered the crucial cytokine for the development of autoimmune diseases such as EAE or collagen-induced arthritis (71,72,73). While IL-23 is responsible for the maintenance of these diverse inflammatory IL-17-producing $CD4^+$ T cells, *de novo* generation depends on the presence of IL-6 and TGF-β *in vitro* (74). On the other hand, IL-27 was shown to suppress the development of T_H17 cells (75).

THE BLOOD BRAIN BARRIER AND THE IMMUNE PRIVILEGE OF THE CNS

The CNS is often referred to as a site of limited immune surveillance. This concept dates back to the seminal work of Medawar (76) and Barker & Billingham demonstrating that allografts fare better in the CNS compared to other tissues (77,78). It was also commonly assumed that the CNS is rare of leukocytes and its "immunologically privileged" status has for long been attributed to the blood-brain barrier (BBB) and the lack of classic lymph vessels. The model of a BBB describes a mechanical diffusion barrier for hydrophilic molecules (including Abs and complement) to selectively separate the blood from the CNS parenchyma. The BBB is formed by specialized tight junctions of endothelial cells of the vessel wall which are surrounded by astroglial endfeet forming the glia limitans (79). The space between the blood vessel and the glia limitans is termed Virchow-Robin space or perivascular space (Figure 4). The function of the BBB is thought to restrict lymphocyte infiltration in order to prevent the development of an inflammatory response and neuronal damage. Infection of peripheral tissues with LCMV in mice usually induces a massive expansion of virus-specific $CD8^+$ T cells, which results in efficient viral clearance (80). When the virus is inoculated directly to the CNS, however, it causes a lethal disease lacking $CD8^+$ T cell infiltration (81,82). Hence in the case of infection, the CNS appears to be a defenseless victim at the mercy of the peripheral immune system to control or end the attack. From an evolutionary point of view, it may in fact be less detrimental for an individual to tolerate certain neurotropic viruses (such as varicella zoster) than the elimination of all infected neurons (83,84).

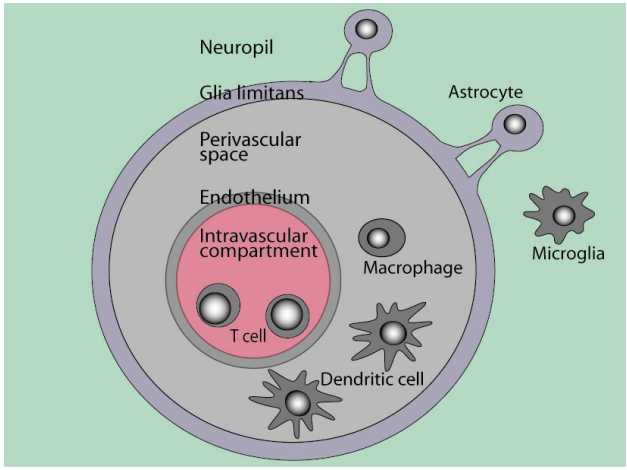

Figure 4: Topography of the perivascular space.
Adapted and modified from Bechmann *et al*. (79,1)

The previous definition of an "immune-privileged" CNS was challenged by various studies. It became apparent that the CNS is within constraints to immune cells in regards to the delicate balance of costs over benefits during neuroinflammation. Leukocytes readily cross brain endothelial cells to reside in perivascular spaces or move on into the CNS parenchyma (85,84,86). It is also established that Ags efficiently drain into cervical LNs via the cribroid plate and perineural sheath of cranial nerves (87). Recent data suggest similar routes for APCs (88,89,90) (see also page 24, "CNS-associated dendritic cells – sentinels at the gateway to the CNS"). The immune-privilege of the CNS is therefore not absolute but crucial to limit neuroinflammation and neurodegeneration in a tissue of vital importance in which regeneration is limited.

ANTIGEN PRESENTATION AND TARGET RECOGNITION IN EAE

After immunization-induced expansion of encephalitogenic T_H cells in the systemic immune compartment, these T cells are now equipped to leave the lymphoreticular environment and invade the bodies' tissues scanning for APCs presenting their cognate Ag. Oligodendrocytes are ultimately the target of such T_H cells. They are the CNS' myelinating cells wrapping their cell membranes around axons to provide nerve insulation for optimal conductivity. However, while oligodendrocyte loss and demyelination are ultimately seen in EAE, oligodendrocytes cannot directly be recognized by CNS invading T cells as they are incapable of expressing MHCII molecules (91). A compulsory third party APC is an absolute requirement for encephalitogenic T_H cells to recognize myelin Ag within the CNS. Cerebral microvascular endothelial cells, which are part of the BBB, are not considered as APCs (92) since they do not constitutively express MHCII molecules and fail to induce T cell proliferation *in vitro* (92,92). Whether astrocytes, the major glial population within the CNS, mediate APC-T cell interaction and activation is a lively controversy (93,94,95). Although astrocytes display the functional capacity to present Ag *in vitro*, their contribution as APCs to the pathogenesis of EAE remains unlikely.

The cell type and location of the APC mediating T cell entry into the CNS has so far remained unknown. CNS-resident cells (microglia) or CNS-associated cells (DCs and

macrophages at the BBB) represent ideal candidates to potentially present myelin epitopes to infiltrating T cells and will be discussed in detail below.

MICROGLIA – SENTINELS OF THE CNS PARENCHYMA

Glial cells were initially defined as the glue (greek: glia) keeping the neurons in the brain together (96). In 1932 the neuropathologist del Rio Hortega described a third type of glial cells in addition to astroglia and oligodendroglia, later named microglia (97). Microglia comprise about 10% of the total glial population in the CNS parenchyma (96) and are in contrast to the other cells residing in the CNS parenchyma not of neuroectodermal but of mesodermic origin. The most widely held hypothesis is that during embryogenesis, early monocyte precursor cells migrate from the yolk sac into the developing CNS where they differentiate into parenchymal microglial cells (98). Unlike the CNS-associated phagocytes of the leptomeninges and the perivascular spaces, microglia are not readily repopulated by bone marrow (BM)-derived monocytes during adulthood (99). But there is evidence for regional turnover at low rates and rapid transformation of blood monocytes into microglia during pathologies (100,84). Microglial cells are regarded as the resident macrophages of the CNS and indeed they share many properties with them, having developed from a common precursor cell. Microglia are rapidly activated from their quiescent state to any pathological change in the CNS such as inflammation, infections, injuries, neurodegeneration and tumors (101). Their ability to respond to a vast array of stimuli is considered vital for the first line of defense of the CNS. Nevertheless their uncontrolled activation, release of inflammatory cytokines (such as IL-1, IL-6 and TNF-α) and neurotoxic molecules in many CNS diseases are implicated to account for unspecific tissue damage.

Microglia are not only involved in phagocytic functions but are also thought to participate in Ag-presentation which is displayed by the rapid upregulation of MHCII and the costimulatory molecules in response to virtually all inflammatory conditions (102,103,104). Microglia, like CNS-associated cells, phagocytose myelin (105,106,107), indicating that these cells could potentially present Ag and activate myelin-specific $CD4^+$ T cells thus causing specific tissue damage.

Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease (TMEV-IDD) serves as an animal model for MS and is characterized by persistent TMEV infections of microglial cells and the chronic progressive $CD4^+$ T cell-mediated demyelination (108). It was shown *in vitro* that microglial cells isolated from TMEV infected mice were activated to efficiently process and present not only endogenous viral epitopes but also exogenous myelin epitopes to $CD4^+$ T cells (109,110).

Human microglia, either immediately *ex vivo* or cultured for several days, have been reported to act as APCs, and to induce both primary and secondary proliferative T-cell responses (111,102). Similar APC potential was also demonstrated for rat microglia isolated from neonatal brains (112,95), but in these studies, extraneural, leptomeningeal and perivascular CNS-associated APCs were not separated from microglia and may contribute to T cell activation. Recent reports have raised the concern whether such neonatally-derived cells faithfully represent the adult population *in vivo*. In fact, microglia isolated from adult mice fail to present Ag to naïve Ag-specific $CD4^+$ T cells (113), but those that are pretreated with IFNγ or isolated from EAE-diseased mice can activate T cell lines in an Ag- and CD86-dependent manner *in vitro* (114).

The activation and differentiation of $CD4^+$ T cells require MHCII recognition and co-stimulation. Studies using adoptive transfer of primed myelin-reactive $CD4^+$ T cells into mice in which APCs can no longer deliver a specific co-stimulatory signal have identified CD40, CD80/CD86 and CD134 (OX40) as functional for reactivation of these T cells in the CNS (115,116). Recent work using BM-chimeric mice pinpointed the primary source of these costimulatory molecules in the CNS. BM-chimeric mice in which CD40 is deficient only in microglia but is intact in CNS-associated cells has revealed that microglial CD40 is most critical to the reactivation of peripherally primed encephalitogenic T cells and the progression of EAE (117). Infiltrating T cells expressing CD40 ligand (CD154) can stimulate microglial CD40 to upregulate IL23 which in turn drives IL-17A expression by these encephalitogenic $CD4^+$ T cells.

Whether microglia participate in the onset and maintenance of MS/EAE or may also play a role in tissue repair via the actions of neurotrophic and immunosuppressive factors is a subject of debate. It is likely that microglia are capable to regulate the

expansion of encephalitogenic T cells within the CNS. CNS-APCs derived from EAE-diseased mice were found to inhibit T cell proliferation through the release of toxic levels of nitric oxide (118). In addition, isolated microglia from EAE mice were shown to induce T cell apoptosis through Fas-Fas ligand interaction (88,119). In line with this findings, many T cells in the CNS of rodents with EAE undergo apoptosis within the parenchyma (120), where they are in contact with microglia.

For a long time, microglia have appeared to be the most capable APCs to initiate and sustain a T cell-mediated immune response within the CNS (121). However until now there has been no *in vivo* evidence for their capacity to act as APCs to allow T cell target recognition.

CNS-ASSOCIATED DENDRITIC CELLS - SENTINELS AT THE GATEWAY TO THE CNS

The "immune privilege" which is essential to guard the CNS from invading immune-mediated damage, does not apply to non-parenchymal regions of the brain such as ventricles, meninges or subarachnoid spaces (88). Foreign tissue grafts are eliminated when they are injected into the ventricles and tumors can be rejected when they are located near the ventricles (122,123). It was shown by Matyszak and Perry that injection of the pathogen Bacillus Calmette-Guerin (BCG) to the meninges, but not the CNS-parenchyma, provokes a typical inflammation and a BCG Ag-specific T cell response (124). These examples clearly show that immunity within the CNS does occur and suggest the presence of specialized APCs at the portals to the CNS mediating immune responses. Indeed, although it has long been thought that the CNS is devoid of professional APCs, DCs and macrophages were found to be present in the healthy human and rat CNS. They are located in the perivascular spaces, the choroid plexus and the meninges but are absent from the CNS parenchyma under physiological conditions (125,126,127). These CNS-associated APCs seem strategically positioned to mediate immune surveillance and interaction with the immune system.

In contrast to microglia, under steady state conditions, CNS-associated cells are continuously replenished by bone marrow-derived monocytes (128,99). Their

numbers drastically increase upon infections, autoimmune diseases or injuries (129,130). These various pathological conditions lead to DC activation and even migration into the CNS-parenchyma. Aloisi *et al.* demonstrated that DCs were recruited into the CNS parenchyma during EAE and hypothesized that they are a fundamental component for the onset and progression of the disease (126). CNS-associated DCs constitutively express high levels of MHCII. Both *in vitro* and *in vivo* studies strongly point to the CNS-associated cells, rather than the microglia, as competent APCs (120). When isolated from adult rodent CNS, they were, in comparison to parenchymal microglia, more efficient in activating both naïve and primed $CD4^+$ T cells (131,88,119).

Using different MHCII restricting T cell elements, Hickey and Kimura could show that perivascular APCs –"considered to be macrophages" are sufficient to provide stimulatory signals for T cells to infiltrate the CNS. However, in order to induce EAE, the rats were required to develop graft vs. host disease (132) and thus changing the inflammatory environment and infiltrates of the CNS.

CNS-associated DCs seem ideally positioned for influencing T cell trafficking to the CNS parenchyma. When they are depleted, lymphocyte invasion into the CNS parenchyma is blocked and EAE is prevented (133). They may engage in *de novo* processing of CNS Ags for *in situ* activation or tolerance induction of effector/memory as well as naïve T cells. In fact, Ag-recognition in perivascular spaces was proposed to be required for T cells to migrate into the CNS parenchyma across the BBB (134). Upon Ag capture in the CNS, CNS-associated APCs are likely to travel into cervical LNs in order to prime T cells. It was shown that Ag-loaded DCs injected into the CNS migrate into cervical LNs where they initiated primary immune responses (90). In patients with relapsing-remitting MS, myelin-ingested APCs were abundant in cervical LNs (135). Moreover, cervical LNs of marmoset and rhesus monkeys immunized with only MOG, contained an accumulation of APCs that had engulfed other myelin antigens, such as MBP and PLP (136).

A recent study in which MS-patients were treated with a monoclonal Ab against very late antigen 4 (VLA4), demonstrates that restricting the CNS' immune surveillance could potentially lead to out-of-control expansion of pathogens such as the normally well controlled JC virus (human polyoma virus) (137). Therefore, under physiological

conditions, CNS-associated APCs are likely to be vital for the maintenance of peripheral tolerance. Under pathological conditions, the action of CNS-associated APCs seems absolutely essential for the immune invasion of the CNS. However, for CNS-mediated autoimmune diseases, definite *in vivo* data supporting the concept that CNS-associated DCs are the culprits to mediate T cell entry are lacking.

PUBLICATIONS

INDUCTION OF PRIMARY T CELL IMMUNITY IN THE ABSENCE OF SECONDARY LYMPHOID TISSUES

Melanie Greter, Burkhard Becher

(submitted)

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS REPRESSED BY MICROGLIAL PARALYSIS

Frank L Heppner, Melanie Greter, Denis Marino,
Jeppe Falsig, Gennadij Raivich, Nadine Hövelmeyer,
Ari Waisman, Thomas Rülicke, Marco Prinz, Josef Priller,
Burkhard Becher, Adriano Aguzzi

Nature Medicine 2005 (3)

DENDRITIC CELLS PERMIT IMMUNE INVASION OF THE CNS IN AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

Melanie Greter, Frank L Heppner, Maria P Lemos, Berhard M Odermatt,
Norbert Goebels, Terri Laufer, Randolph J Noelle, Burkhard Becher

Nature Medicine 2005 (2)

Induction of primary T cell immunity in the absence of secondary lymphoid tissues

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Subcutaneous immunization delivers antigen (Ag) to local Ag-presenting cells which subsequently migrate through afferent lymphatics into draining lymph nodes. Here, they initiate the activation and expansion of lymphocytes specific for their cognate Ag. In higher vertebrates, the structural environment within secondary lymphoid tissues (SLTs) is considered essential for the initiation of adaptive immunity. We could demonstrate, that the immunodeficiency observed in such alymphoplastic mice results exclusively from the impact of the genetic lesion on immunity rather than from the lymphoreticular malformations. Surprisingly, we discovered that in mice lacking all SLTs, subcutaneous immunization with foreign as well as auto-Ag can initiate productive T cell immunity as well as autoimmune disease respectively. While B cell maturation is highly dependent on the topography of SLTs, potent T cell priming can occur completely independent of lymphoreticular structures. Our data point towards the adult liver as a potential priming site for T cells after subcutaneous immunization.

SLTs are highly organized structures with defined compartments consisting of B cell areas (cortex containing germinal centres (GCs)) and T cell areas (paracortex in lymph nodes (LNs) and periarteriolar lymphoid sheaths in the spleen). These distinct locations support the rapid circulation and concentration of Ag and the interaction of Ag-presenting cells (APCs) with lymphocytes. Prevailing dogma dictates that only if competent APCs transport the Ag into SLTs, an adaptive immune response is initiated; otherwise the Ag is ignored by the immune system [1, 2]. For the initiation of humoral immunity, the formation of B cell follicles and GCs is a prerequisite. GCs comprise networks of follicular dendritic cells (FDCs) which play a vital role in the generation of a high affinity antibody (Ab)-response. However, in contrast to the cortex, T cell areas, where T cells encounter mature APCs, are structurally rather ill-defined. While intravital confocal microscopy has provided compelling evidence for the capacity of SLTs to host T cell priming [3, 4], definitive data supporting their absolute requirement for functional T cell priming does not exist.

Alymphoplasia (*aly/aly*) mice are characterized by a complete lack of LNs and peyer's patches and structural alterations of the spleen and thymus due to a point mutation in the NFκB-inducing kinase (NIK) [5]. NIK is vital for the initiation of the non-canonical NFκB cascade, which appears to play a discrete role in the function of CD40 and lymphotoxin-β receptor (LTβR) signaling in some cell types[6-8]. *Aly/aly* mice display impaired Ab responses and cannot reject allogeneic grafts or tumors [9-11]. The lack of LNs is considered to be responsible for this apparent immunodeficiency [10, 12, 13]. The developmental deficits in *aly/aly* mutants are readily explained by the requirement of NIK in LTβR signaling. LTβR$^{-/-}$ mice display similar developmental defects as do *aly/aly* mice or NIK$^{-/-}$ mice [8, 14]. Others argued that the immunodeficiency in *aly/aly* mice could not be explained solely based on their developmental malformations [15].

Here we demonstrate that immunization-induced T-cell priming and cell-mediated autoimmunity can be induced even in the absence of conventional SLTs. Conversely, B-cell maturation and Ab-class switching is structure-dependent and is completely abrogated in mice lacking SLTs indicating that modern mammalian B cells rely on the topography of dedicated lymphoid tissues, while T lymphocytes retain the capacity to recognize Ag in a structure-independent fashion.

RESULTS

T cell priming and DTH reactions after subcutaneous immunization with protein Ag in the absence of LNs

We wanted to elucidate the impact of the *aly* mutation on the development of a T cell-mediated immune response induced by subcutaneous (s.c.) immunization. We used Keyhole limpet hemocyanin (KLH) as a model foreign Ag to elicit delayed-type hypersensitivity (DTH) responses. *Aly/aly* as well as *aly/+* mice (the latter developing normal SLTs as NIK is not haplo-insufficient) were immunized with KLH and 11 dpi, they were challenged by intradermal injection with KLH into the ear. As illustrated in Fig. 1a, *aly/aly* mice were able to mount a solid DTH reaction measured by ear swelling, which was only marginally lower than in *aly/+* mice. However, in contrast to ear-swelling, which is indicative of a T cell response, *aly/aly* mice did not mount Abs against KLH when compared to *aly/+* mice demonstrating that the development of a humoral immune response is ablated in the absence of lymphoreticular structures (Fig. 1b). We could reproduce functional DTH responses using other Ags including Ovalbumin and myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}).

In order to quantify the expansion of Ag-specific T cells in *aly/aly* mice, CFSE-labeled T cells derived from TcR transgenic mice (2D2) [16] were adoptively transferred into either *aly/aly* or *aly/+* mice prior to immunization with their cognate Ag (MOG_{35-55}). After 4 days, splenocytes were analyzed for T cell expansion by flow cytometry (Fig. 1c). Although Ag-specific T cell expansion can be observed in *aly/aly* mice, primed T cells undergo fewer cell divisions in *aly/aly* mice than in *aly/+* mice. In support of the notion that this delayed response could be attributed to the lack of LNs in *aly/aly* mice, in alymphoplastic $LT\alpha^{-/-}$ mice, antiviral T and B cell responses also display delayed kinetics [17]. Similar results were obtained with OVA-TcR transgenic cells (OTII) transferred into *aly/aly* and *aly/+* mice (not shown).

The *aly* mutation prevents the development of autoimmune disease

We next wanted to assess whether the unexpected observation of effective T cell immunity in mice lacking LNs can be reproduced in a complex T cell-mediated autoimmune response. Experimental autoimmune encephalomyelitis (EAE) is a T_H cell-mediated demyelinating autoimmune disease of the central nervous system (CNS) and serves as the animal model for multiple sclerosis. We have recently shown that *aly/aly* mice are susceptible to EAE induced by adoptive transfer of MOG_{35-55}-specific

T cells, indicating that the migration of fully primed pathogenic T cells to SLTs is not a prerequisite event prior to their invasion of the CNS [18]. To assess the role of SLTs in T cell priming, we induced EAE in *aly/aly* or *aly/+* mice by active s.c. immunization with MOG_{35-55} emulsified in CFA. Fig. 1d shows that *aly/aly* mice are completely resistant to EAE, compared to *aly/+* control mice indicating that pathogenic T cells cannot be raised in *aly/aly* mice. To verify this notion, *aly/aly* and *aly/+* mice were immunized s.c. with MOG_{35-55}. 11 dpi, splenocytes were harvested, MOG_{35-55}-reactive cells were expanded *in vitro* and subsequently transferred into *aly/aly* as well as *aly/+* recipients. Fig. 1e shows that only cells derived from *aly/+* donors were able to induce disease regardless whether the recipients had SLTs (*aly/+*) or not (*aly/aly*). However, MOG-reactive T cells derived from *aly/aly* donors were not pathogenic and did not mediate CNS-inflammation.

Autoimmune disease develops in the absence of SLTs after s.c. immunization with auto-Ag

The fact that *aly/aly* mice do not develop autoimmune disease could be explained by their inability to prime self-reactive T cells i) due to the lack of LNs as suggested by others [12, 19], or ii) by a direct impact of the NIK mutation on immune cells [20, 21]. In order to define whether the developmental malformation in *aly/aly* mice is responsible for their resistance to develop EAE, we generated a series of bone marrow (BM)-chimeric mice. To restrict the NIK mutation to the hematopoietic system, lethally irradiated *aly/+* mice were injected with BM stem cells from *aly/aly* donor mice *(aly/aly→aly/+)*. Conversely, to conserve the developmental structural defects, without the NIK-lesion of the hematopoietic compartment, *aly/aly* mice were reconstituted with BM-cells of normal *aly/+* donors (*aly/+→aly/aly)*. Spontaneous development of lymphoid tissues in *aly/aly* recipients upon reconstitution was expectedly not detected. Surprisingly, we discovered that *aly/+→aly/aly* BM-chimeras were fully susceptible to EAE after s.c. immunization with MOG_{35-55} (Fig. 2a), clearly demonstrating that s.c. immunization can mount a productive T cell-driven autoimmune response even in the absence of draining LNs. Using the reciprocal approach, by generating *aly/+→aly/+* ($NIK^{+/+}$ immune system and normal SLTs) as well as *aly/aly→aly/+* BM-chimeras (NIK-deficient immune system and normal SLTs), we found that the NIK mutation lead to EAE resistance even when the lymphoreticular compartment is unperturbed (Fig. 2b). In support of this, we found

that unmanipulated LTβR$^{-/-}$ mice, which also lack all LNs but have normal NIK function, are also fully susceptible to EAE (Supplementary Fig. 1a).

The formation of IFNγ and IL-17 secreting auto-reactive T cells has been demonstrated to be a prerequisite for the development of autoimmunity [22]. To specifically measure the impact of NIK on the development of Th-17 cells, LNs of MOG-immunized *aly*/+→*aly*/+ and *aly/aly*→*aly*/+ BM-chimeras were analyzed for IL-17 and IFN-γ production by enzyme-linked immunospot analysis (Elispot). *Aly/aly*→*aly*/+ mice display a substantial reduction in IL-17 and IFN-γ producing cells compared to the control mice *aly*/+→*aly*/+ (Fig. 2c) indicating that the resistance to EAE in the absence of NIK is due to the reduced generation of auto-reactive IFNγ and IL-17 secreting cells. Thus, the loss of NIK function impairs the capacity of *aly/aly* mice to generate pathogenic Th cells regardless of their structural defects.

Our findings raise the issue that the immunodeficiency of *aly/aly* mice is disconnected from their structural malformations and due to the impact of NIK-signaling on immunity. Given the dogma that immunization-induced adaptive immunity requires the presence of SLTs, it is feasible that the spleen in *aly/*+→*aly/aly* BM-chimeras compensates for the absence of LNs and serves as an alternative site for T cell priming. In order to test this notion, we splenectomized *aly/*+→*aly/aly* BM-chimeras (*aly/*+→*aly/aly*^{spl}) 14 days prior to the induction of EAE. Upon immunization with MOG, *aly/*+→*aly/aly*^{spl} mice developed EAE with the same disease severity as control mice (Table 1). We noted a slight delay in disease onset when all SLTs are absent. Histology of CNS tissue however revealed no significant difference between *aly/*+→*aly/*+ and *aly/*+→*aly/aly*^{spl} mice (Supplementary Fig. 1b). These data conclusively reveal that potent cellular immunity can be induced successfully upon s.c. immunization with Ag in CFA even in the absence of SLTs.

Ab and GC formation is dependent on the structural support of SLTs

The development of B cell activation and IgG class switching is generally held to require dedicated lymphoreticular structures and GC formation. *Aly/aly* mice are known for their inability to form GCs and intrinsic B cell defects, associated with the mutation in NIK, lead to impaired Ab responses and class switching [7]. Again, to separate the structural malformations and the NIK lesion of the hematopoietic system, we found that anti-MOG Abs are virtually absent in mice without LNs (either

aly/+→aly/aly or *aly/+→aly/aly*^{spl}). Yet, control mice (*aly/+→aly/+* and *aly/+→aly/+*^{spl}) elicit high anti-MOG Ab titers (Fig. 2d). Analysis of isotype subtypes revealed that in splenectomized alymphoplastic mice, anti-MOG IgM could be detected, while class switching to IgG did not occur (Fig. 2e-f). Anti-MOG IgA levels were low in all groups (Fig. 2g). Taken together, for B cell activation, the generation of high-affinity Igs and class switching, highly organized SLTs are obligatory.

S.c. delivered Ag is transported into the liver in the absence of SLTs

Since the loss of SLTs does not hinder the development of T cell immunity, we wanted to determine at which alternative site T cell priming could take place and to which organ the Ag travels from the site of immunization (s.c.). Therefore *aly* BM-chimeras were injected s.c. with FITC-Dextran-coupled microspheres emulsified in CFA. 7dpi various organs (BM, thymus, CNS, liver, lung, blood, gut and in control mice spleen and LNs) were isolated and analyzed for the presence of fluorescent cells by flow cytometry. Fig. 3a shows that in control mice (*aly/+→aly/+*) FITC positive APCs were exclusively detected in LNs upon s.c. immunization. However, in *aly/+→aly/aly*^{spl} BM-chimeras lacking SLTs, APCs carrying FITC beads migrate primarily to the liver and not the other organs analyzed. Interestingly, we further observed that upon s.c. immunization we reproducibly obtained a significantly greater number of mononuclear cells from the livers of *aly/+→aly/aly*^{spl} mice when compared to *aly/+→aly/+* or naïve mice (Fig. 3b).

In order to determine whether lymphoid-like structures can be found in the liver, we analyzed the liver of MOG-immunized mice by histology (d7). We found that livers of *aly/+→aly/aly*^{spl} BM-chimeras were severely inflamed in comparison to *aly/+→aly/+* control mice (Fig. 4a-c). Detailed histological analysis displays dendritic cells (DCs) in close proximity to T cells in the infiltrated areas of the liver (Fig. 4c). Few FDCs, PNA^{+} cells and HEVs (CD62LL) were detected, which is in contrast to the described structured neogenesis of tertiary lymphoid structures. In the liver, we could also detect the accumulation of Ag-specific TcR Tg T cells (Supplementary Fig. 2).

Induced destruction of lymphoid structures inhibits B cell, but not T cell activity

While SLTs require the presence of a cellular *Anlage* to form LNs and follicular structures, tertiary lymphoid development can be induced by chronic inflammation in

solid tissues (for review see [23]). To determine whether newly formed tertiary lymphoid tissues (TLTs) can serve to propagate T cell expansion, we treated mice with LTβR-Fc fusion protein, which has been shown to efficiently disrupt follicular structures in SLTs and prevents the formation of TLTs [24]. In agreement with the report by Gommermann *et al.* [25], Fig. 4d demonstrates that LTβR-Fc treated mice are fully susceptible to EAE and display a similar clinical score as control mice. The splenic architecture of diseased mice treated with LTβR-Fc displays that PNA^+ GC clusters are disrupted and FDCs are absent, verifying that LTβR-Fc fusion proteins completely prevented follicle formation (Fig. 4f). Anti-MOG Ab titers of diseased LTβR-Fc treated mice further revealed diminished levels of anti-MOG Abs (Fig. 4e and Supplementary Fig. 3). This however is not expected to impact on EAE in C57BL/6 mice, as they develop disease completely independent of B cells or anti-MOG Abs, demonstrated by the susceptibility of B cell deficient mice to EAE [26, 27].

DISCUSSION

A fundamental immunological dogma holds that primary adaptive immune responses are initiated in SLTs[1]. We wanted to investigate whether LNs are required for the development of T cell-mediated immunity induced by subcutaneous (s.c.) immunization. We found that a*ly/aly* mice could mount a substantial DTH response after s.c. immunization with protein or peptide Ag. However, they are completely resistant to developing EAE induced by s.c. immunization with MOG_{35-55}/CFA. Our results reveal that the lymphoreticular malformation is not the underlying cause of the immune-defects observed in *aly/aly* mice. The data presented here clearly demonstrate that mice lacking all SLTs are fully susceptible to EAE. Hence T cell priming and cellular immunity can be initiated in the absence of SLTs.

We show that lesioning the function of NIK prevents the generation of pathogenic self-reactive TH17 cells and that it is the impaired NIK signalling in immune cells, rather than the absence of SLTs which prevents autoimmune disease from developing. In contrast to T cell priming, mice lacking SLTs (*aly*/+→*aly/aly*^{spl} BMCs) or mice treated with LTβR-Fc, which disrupts lymphoreticular structures, are not capable to mount Ag-specific Abs and isotype switching. Thus, for B cell activation and the generation of high affinity Abs, the structural and functional support from dedicated SLTs is obligatory.

Previously, Schirrmacher *et al*. suggested that the BM could be an alternative priming site for T cells[28]. They showed that blood-borne Ag can drive the expansion of adoptively transferred TcR Tg T cells independent of the presence of SLTs. Given that the priming requirements of a large population of adoptively transferred monoclonal TcR Tg T cells are likely less stringent than for endogenous naïve T cells, we extended this experiment to include the endogenous polyclonal Ag-specific population. Additionally, while blood-borne Ag, can reach virtually every tissue though the blood stream, we delivered an Ag depot s.c. and failed to observe any diffusion away from the injection site. In contrast, we found that the Ag is carried by local APCs from the site of immunization into the liver when SLTs are not available. In addition, we could demonstrate that s.c. immunization of mice lacking SLTs results in the accumulation of inflammatory T cells in the liver, suggesting that the liver can serve as a potential site of T cell priming when dedicated LNs are absent. While this immune function is not traditionally attributed to the adult liver, the fetal liver is a primary lymphoid organ hosting early hematopoiesis. We cannot claim that the liver

is the only potential site to host SLT-independent T cell priming, but our findings suggest that the adult liver has the potential to "remember" this function.

Interestingly, during evolution, the appearance of adaptive immunity was brought about by the emergence of RAG in jawed fish [29-31]. RAG mediates somatic recombination and is required for the formation of both B & T cell receptors, which appear to have emerged simultaneously during evolution. However, while the adaptive immune system is well developed in the oldest jawed vertebrates (cartilaginous fish e.g. sharks), Ig-class switching and GC formation are lacking. Class-switching only appeared at the time of the divergence of amphibians [32]. The fact that cell-mediated immunity evolved earlier than modern humoral immune responses corroborates our discovery that T cell-mediated immunity can function outside of dedicated lymphoreticular structures. In summary, we found that B cells are dependent on the topography of dedicated lymphoid tissues, while T lymphocytes retain the capacity to recognize Ag in a structure-independent fashion. This finding has obvious implications for our understanding of adaptive immunity and vaccination. As for the development of autoimmune diseases, our findings show that self-reactive T cells may not need to be primed in tissue-draining LNs, but could occur at the inflammatory site or even in organs distant to the target tissue.

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Table 1 Mice devoid of SLTs are fully susceptible to EAE.

BM-chimeras	Incidence (%)	Mean day of disease onset*	Mean maximal clinical score*
aly/+ → *aly*/+	94.7 (18 of 19)	11.4	3.0
aly/+ → *aly*/+^{spl}	81.8 (9 of 11)	11.7	3.0
aly/+ → *aly*/*aly*	73.7 (14 of 19)	13.5	3.3
aly/+ → *aly*/*aly*^{spl}	64.7 (11 of 17)	15.7	3.4
aly/*aly* → *aly*/+	0 (0 of 10)	-	-

* of diseased animals

FIGURE LEGENDS

Figure 1 DTH responses can emerge in the absence of SLTs. **(a)** DTH responses were induced by s.c. immunization with KLH in *aly/aly* and *aly/+* mice. 11dpi, the mice were challenged by intradermal KLH (black bars), or PBS (grey bars) injection into the ear. Swelling was measured 24h post challenge and shown is the increase of ear swelling over baseline of a representative of 3 independent experiments (n ≥2 /exp). **(b)** Sera was collected from KLH-immunized *aly*/*aly* (▽) and *aly*/+ mice (◆) mice on 12 dpi and analyzed for the presence of total anti-KLH Abs by ELISA. Results are representative of 3 independent experiments ($n \geq 2$ mice/group). **(c)** FACS analysis of splenocytes derived from *aly/aly* and *aly*/+ mice transferred with CFSE-labeled 2D2 ($Va3.2^+$) cells and immunized s.c. with MOG_{35-55}. Results are representative of 2 individual experiments (n ≥ 2 mice/group). **(d)** EAE was induced by active immunization with MOG_{35-55}/CFA in *aly*/*aly* (▽) and *aly*/+ mice (◆). **(e)** EAE was induced by adoptive transfer of pathogenic T cells derived from *aly/aly* or *aly*/+ donors into *aly*/*aly* or *aly*/+ recipients. *aly*/+ into *aly*/+: ▲, *aly*/+ into *aly*/*aly*: Δ, *aly/aly* into *aly*/+: ●, *aly/aly* into *aly*/*aly*: ○. Shown is a representative of two individual experiments (n ≥ 5 mice/group).

Figure 2 SLTs are crucial for Ab affinity maturation and isotype switching. (**a,b**) EAE progression in BM-chimeras immunized s.c. with MOG_{35-55}. **(a)** *aly*/+→*aly*/+: ▲, *aly*/+→*aly*/*aly*: ●. **(b)** *aly*/*aly*→*aly*/+: □, *aly*/+→*aly*/+: ▲**.** **(c)** LN-derived cells were obtained from *aly*/*aly*→*aly*/+ (black bars) and *aly*/+→*aly*/+ (grey bars) BM-chimeras 21 dpi with MOG_{35-55}/CFA and rechallenged *in vitro* with 50μg/ml MOG_{35-55} peptide to reveal IFNγ and IL-17 secreting cells using Elispot. Shown is the average of 3 mice/group (± SEM). **(d-g)** Titers of anti-MOG Abs determined from sera of diseased BM-chimeras by ELISA. **(d)** Total anti-MOG Igs. (**e)** Anti-MOG IgG. (**f)** Anti-MOG IgM. (**g)** Anti-MOG IgA. *aly*/+→*aly*/+: ■, *aly*/+→*aly*/$+^{spl}$: □, *aly*/+→*aly*/*aly*: ▲, *aly*/+→*aly*/aly^{spl}: Δ. Shown is the average of 3 mice/group (± SEM).

Figure 3 Ag-laden APCs migrate to the liver in the absence of SLTs. **(a)** *Aly* BM-chimeras were injected s.c. with FITC-coupled microspheres and various organs were analyzed by FACS for the presence of $FITC^+$ APCs 7dpi. Data represent one of 3 individual experiments. **(b)** Mean number (± SEM) of mononuclear cells isolated

from the livers of MOG-immunized BM-chimeras and naïve wt mice. Statistical significance was assessed using an unpaired Student *t*-test. ns: not significant, $p > 0.05$.

Figure 4 Accumulation of MOG-reactive T cells occurs in the liver in mice lacking SLTs. **(a,b)** Liver cryosections from *aly* BM-chimeras immunized s.c. with MOG_{35-55} (d7) were stained with H&E. Bar: 500 µm. **(c)** Higher magnification image indicated by the region square in (B) stained with H&E and mAbs against CD3 and CD11c. Bar: 100 µm. **(d)** EAE was induced by s.c. immunization with MOG_{33-55} in LTβR-Fc treated (▲) and untreated (□) wt mice. Results are representative of 2 independent experiments ($n \geq 5$ mice/group). **(e)** Total anti-MOG Ab ELISA from sera of diseased LTβR-Fc (▲) and untreated wt mice (□). Shown is the average of 4 mice per group ± SEM. **(f)** Cryosections of spleen obtained from immunized LTβR-Fc treated and untreated wt mice stained with PNA. Bar: 200 µm.

Supplementary Figure 1 (a) EAE was induced in $LT\beta R^{-/-}$ mice and disease development was assessed as described. $LT\beta R^{-/-}$ (Δ) mice are fully EAE susceptible when compared to C57BL/6 (■) wt mice (n=6).
(b) H&E stainings of spinal cord sections of diseased *aly*/+→*aly*/+ and *aly*/+→*aly*/aly^{spl} BM-chimeras. Lower row represents higher magnification of the insert in upper row. Bar in upper row: 200 µm and in lower row: 50 µm.

Supplementary Figure 2 (a) *aly*/+→*aly*/+ and *aly*/+→*aly/*aly^{spl} BM-chimeras were injected with $20x10^6$ CFSE-labeled splenocytes from SMARTA/Thy1.1 TcR transgenic mice and immunized s.c. with p11 peptide of LCMV gp61 emulsified in CFA. 5 dpi LNs (only in *aly*/+→*aly*/+) and liver-invading cells were analyzed by flow cytometry by gating on $Thy1.1^+$ cells (n = 3 mice/group). **(b)** 4 dpi with p11, liver sections were stained with αThy1.1 to reveal Ag-driven T cells accumulating in the livers of *aly*/+→aly/aly^{spl} mice. Scale bar: 500 µm.

Supplementary Figure 3 Anti-MOG Ab ELISA from sera of diseased LTβR-Fc treated (▲) and untreated (□) mice (d17). **(a)** Anti-MOG IgG. **(b)** Anti-MOG IgM. **(c)** Anti-MOG IgA. Shown is the average of 4 mice per group (± SEM).

MATERIAL AND METHODS

Mice. C57BL/6 mice were purchased from Harlan Laboratories. Alymphoplasia (*aly/aly*) mice were bred in house under SPF-conditions. Heterozygous *aly* (*aly*/+) mice were used as controls for homozygous *aly* mice (*aly*/*aly*). 2D2 (MOG-TCR Tg) were provided by V. Kuchroo (Harvard Medical School, Boston, Massachusetts), SMARTA mice (carrying an MHC II restricted TcR specific for gp61 of LCMV) were obtained from R. Zinkernagel (University Hospital Zurich, Zurich, Switzerland), LTβR$^{-/-}$ mice were provided by A. Aguzzi and M.Heikenwalder (University Hospital Zurich, Zurich, Switzerland) and OTII mice were purchased at Jackson Laboratories. All mice were bred in house under SPF-conditions. BM-chimeras were generated as described previously [33, 34]. Mice were splenectomized as described previously [18]. Animal experiments were approved by the Swiss veterinary Office (68/2003, 70/2003 and 13/2006).

LTβR-Fc treatment. C57BL/6 mice were injected i.p. with 100 μg of human LTβR-Fc Fusion protein (generously provided by Dr. Yang Xin Fu, University of Chicago) or 100 μg human IgG control (Bioexpress) 7 days prior to immunization with MOG and weekly thereafter.

Induction of EAE. MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) was obtained from GenScript. EAE was induced as described previously [35] with the modification that BM-chimeras were generally not boosted with pertussis toxin. For adoptive transfer MOG-reactive lymphocytes were generated as described [33]. Each time point shown is the average disease score of each group ± SEM.

Delayed-type hypersensitivity (DTH) assay. Mice were immunized s.c. with 100 μg/flank of MOG_{35-55} peptide , or KLH (Sigma) emulsified in CFA. 11dpi, mice were challenged by injecting 10 μg/10μl of MOG_{35-55} peptide, KLH, PBS into the dorsal surface of the ear. DTH responses were determined by measuring the ear thickness using a calliper micrometer (Mitutoyo) 24 h after challenge and Δ ear swelling was established by the increase in ear thickness over baseline (pre-challenge ear thickness).

Lymphocyte isolation. Mice were euthanized with CO_2 and various organs were removed to isolate lymphocytes: Spleen, LNs, thymus and lung were homogenized

and bone marrow cells were isolated by flushing the bones with PBS. The cells were strained through a 100 μm nylon filter (Fisher) and washed. Blood, BM and splenic red blood cells were lysed. For isolating hepatic non-parenchymal cells, the liver was incubated with DNase (0.5mg) / Liberase (1mg/ml) (Roche) for 30 min at 37°, homogenized and centrifuged at RT for 2 min. The supernatant was then centrifuged at 1500 rpm for 10 min and the pellet was resuspended in 30% Percoll (Pharmacia) and centrifuged at 12000 rpm for 30 min at 4C°. The interphase cells were collected and washed. For isolating intestinal lymphocytes, intestines were opened longitudinally, washed and cut into small pieces. Tissues were then incubated with DNase/Liberase and lymphocytes were isolated using a percoll gradient as described above. Isolation of CNS lymphocytes has been described previously [33, 35].

Proliferation assay. Mice were injected with $20x10^6$ CFSE (carbofluorescein diacetate succinimidyl ester)-labeled (Invitrogen-Molecular Probes) (10μM) 2D2 splenocytes and immunized s.c. with 200 μg of MOG_{35-55} emulsified in CFA. 4 dpi, mice were sacrificed and spleens were analysed by FACS for the proliferation of $CD4^+$ T cells using the 2D2 specific TCR Va3.2 antibody.

Histology and flow cytometry. Tissues were freshly snap-frozen in liquid nitrogen. To determine infiltration of inflammatory cells, tissue sections were stained with hematoxylin and eosin (H&E) or with the following mouse-specific antibodies (Abs) as previously described [18]: anti-CD11c (Jackson ImmunoResearch Labs) and anti-CD3 (BD-Pharmingen). GCs cells were stained with peanut agglutinin (PNA; Vector Laboratories).

For FACS analysis the following Abs were used: anti-CD11c, anti-CD11b, anti-Va3.2, anti-Thy1.1 (BD). The cells were analyzed using a FACS-Canto (BD) with Cell-Diva™ software. Post-acquisition analysis was performed using FLOWJO™ software. To trace the distribution of Ag after immunization, mice were injected s.c. with 200 μl Fluoresbrite™ Carboxylate YG 1.0 micron microspheres (Polysciences) emulsified in CFA supplemented with 5 mg/ml of Mycobacterium tuberculosis. 7 days after injection mice were euthanized with CO_2 and organs removed to isolate lymphocytes as described above. Single cell suspensions were analyzed by FACS for the presence of $FITC^+$ cells.

Enzyme-linked immunosorbent assay (ELISA). Plates were coated with 10µg $rMOG_{1-121}$ or KLH in 0.1 M $NaHCO_3$ (pH 9.6) at 4°C overnight and blocked with 1% (w/v) bovine serum albumin (BSA). Diluted sera were incubated for 2h at RT. After washing, peroxidase-conjugated antibodies to mouse Ig's, IgG, IgA, IgM (Sigma) were added (1:1000 diluted) and incubated for 1h at RT. Plates were washed and chromogen (Biosource) was added. Absorbance was measured on a micro plate reader (450nm) (Bio-Rad).

Enzyme-linked immunospot analysis (Elispot). 2 x 10^5 cells were plated in medium containing 10% FCS and MOG_{35-55} 50µg/ml in 96 well plates (Millipore) coated with the capture Ab against either IFNγ or IL-17A[35]. Elispots were revealed as described previously[35] and subsequently analyzed on an Elispot reader (CTL immunospot).

Figure 1

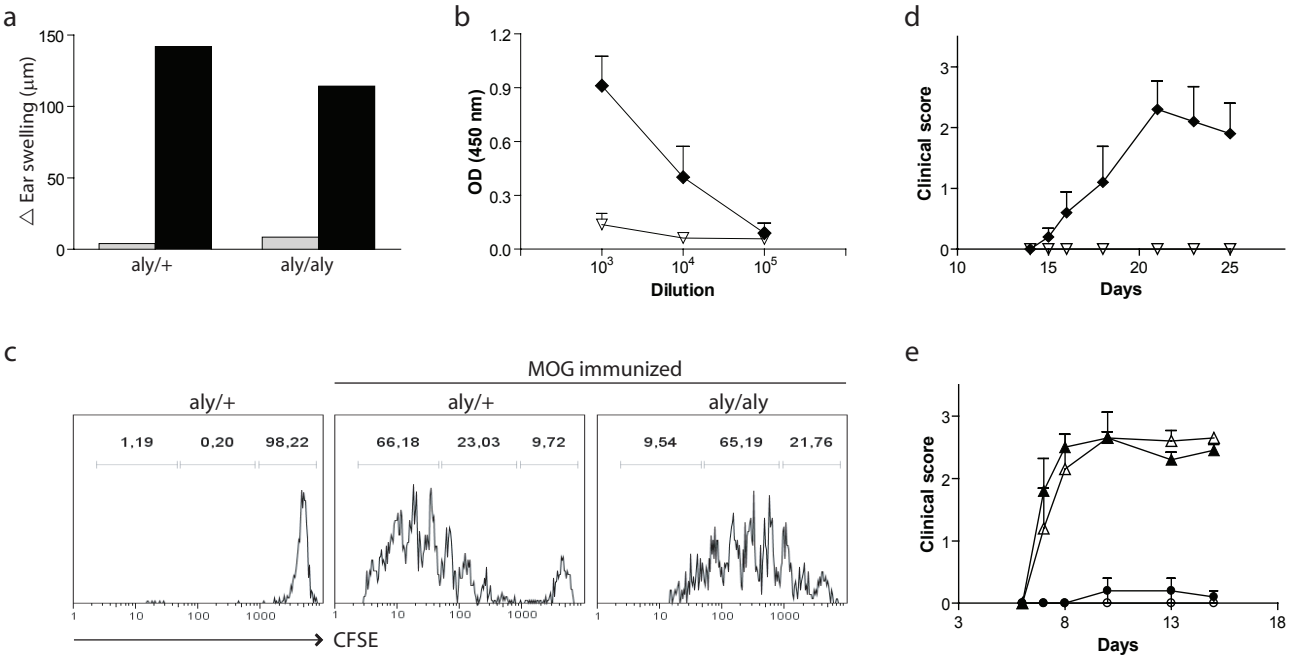


Figure 2

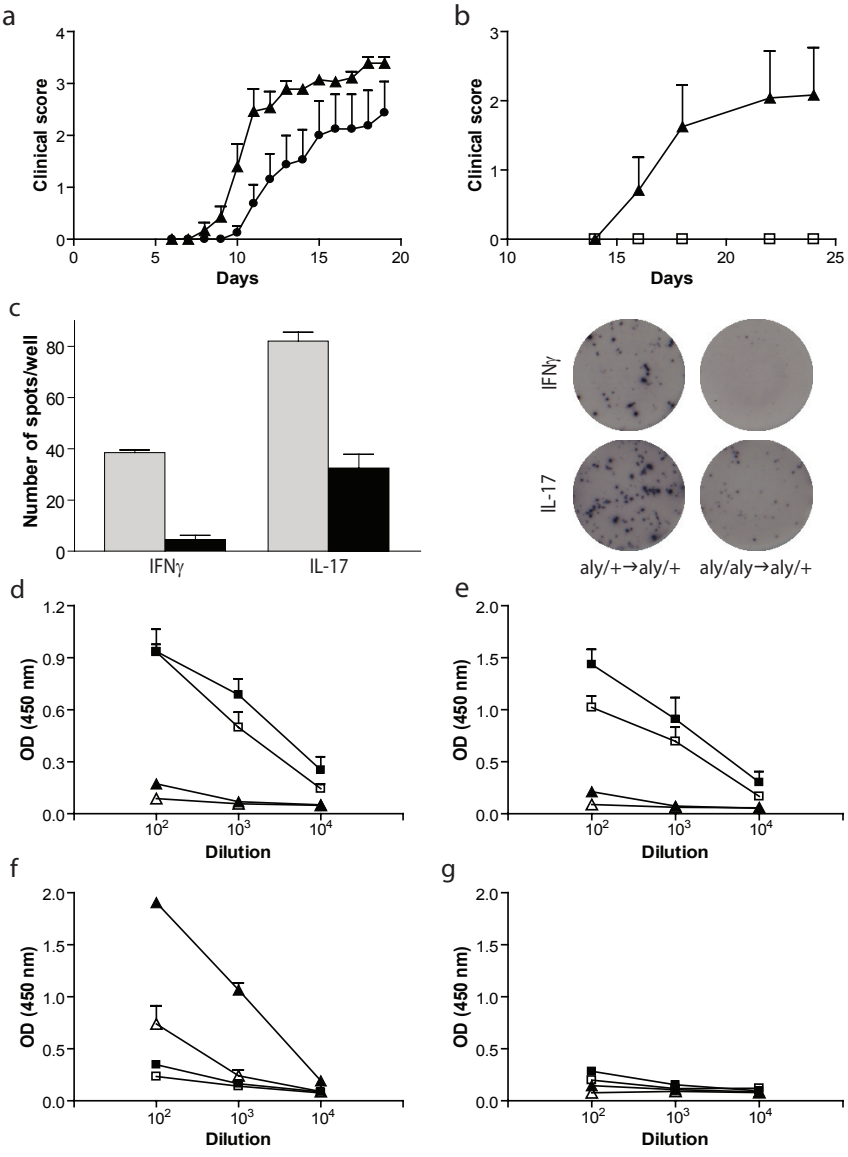


Figure 3

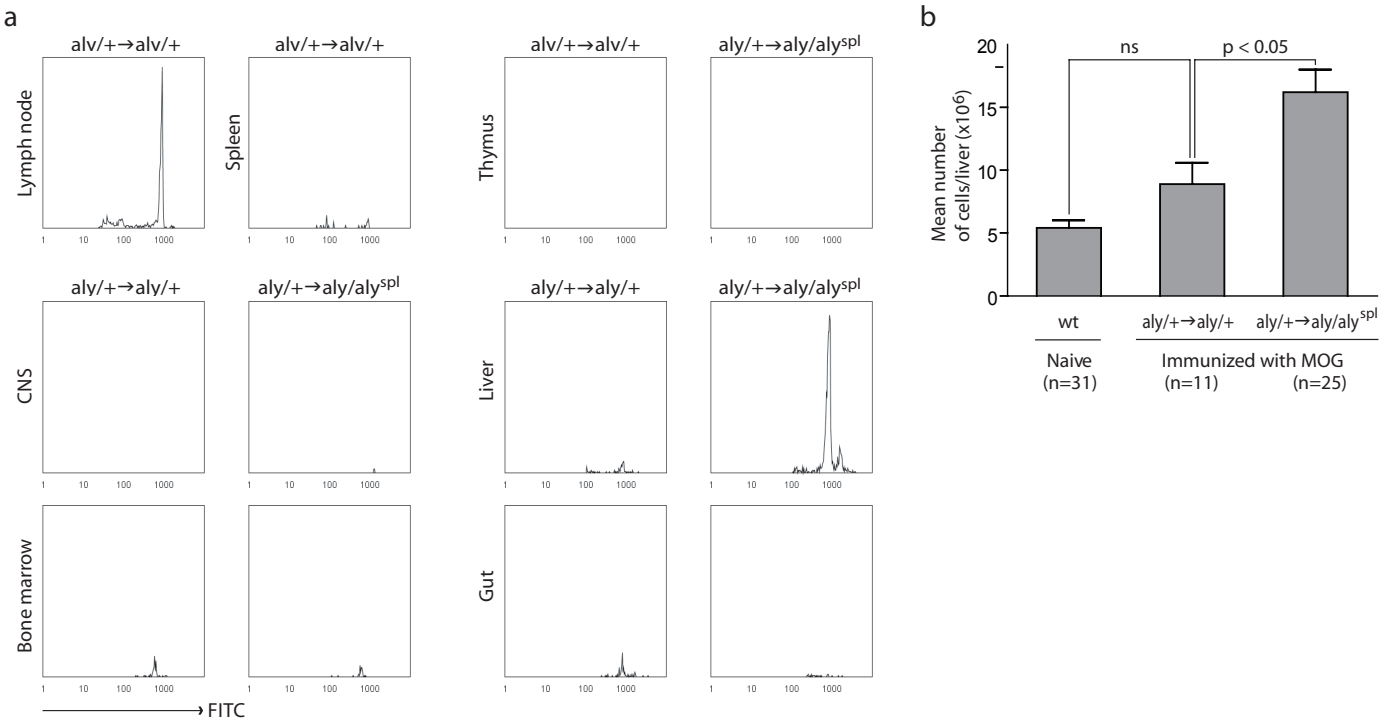
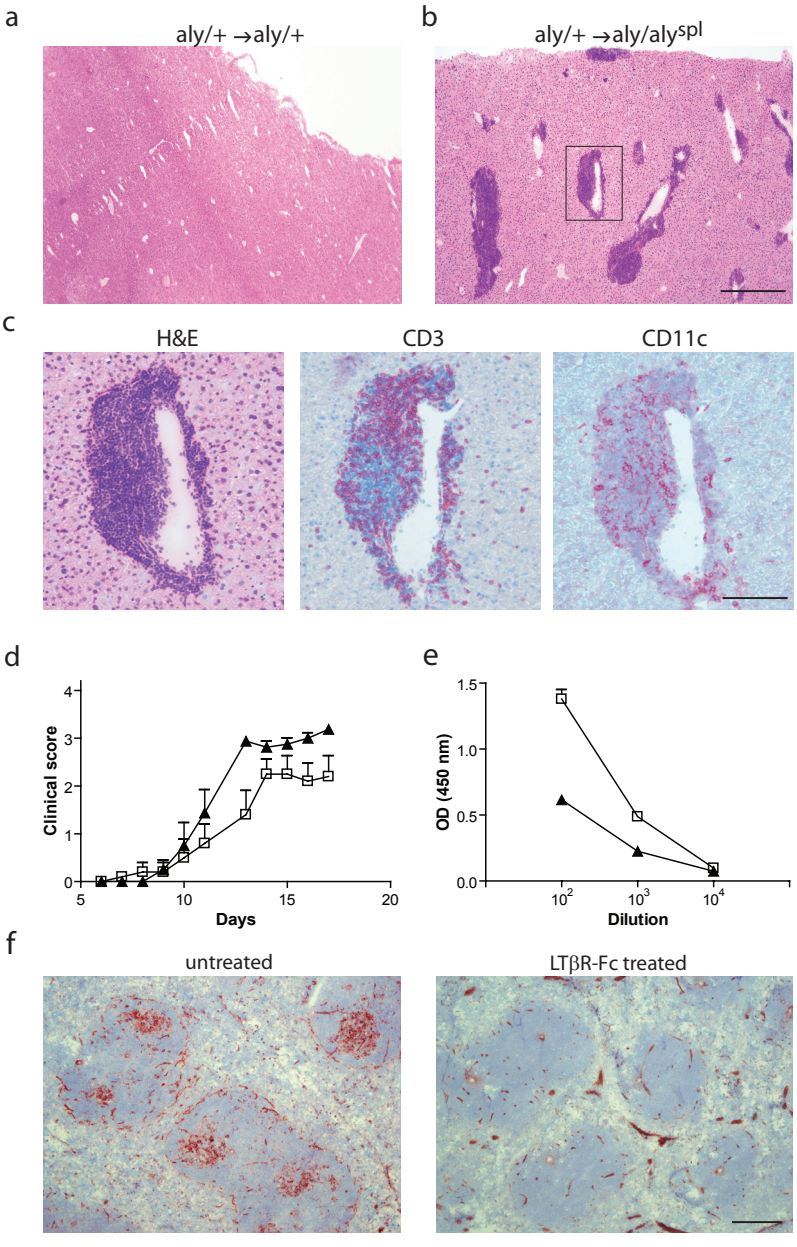
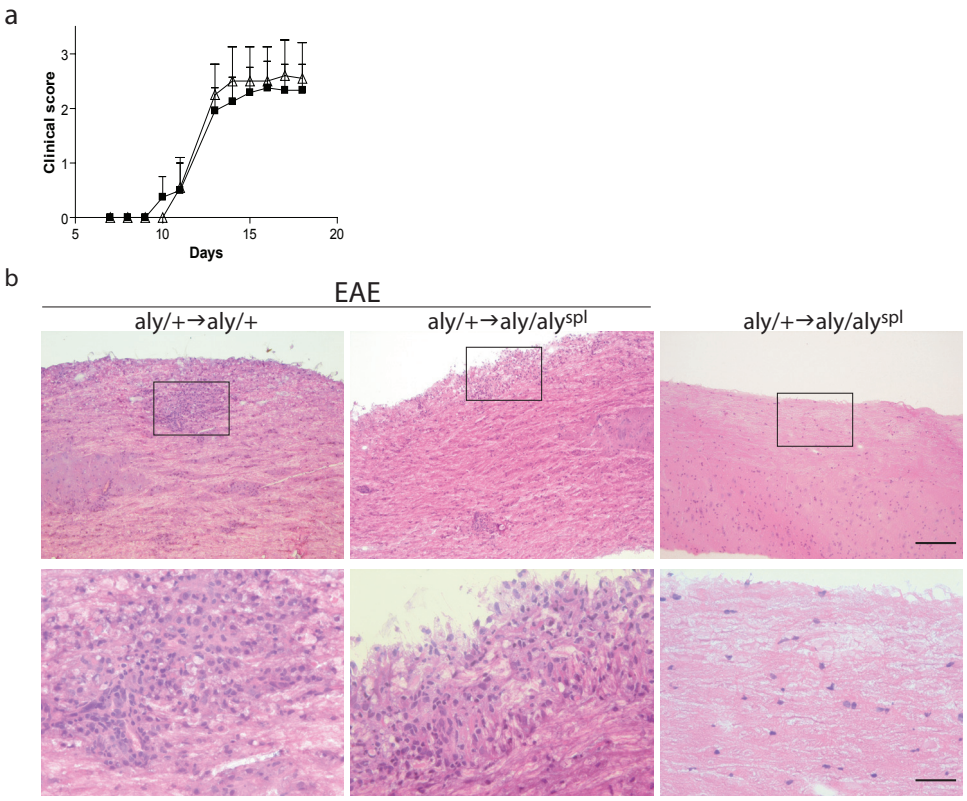


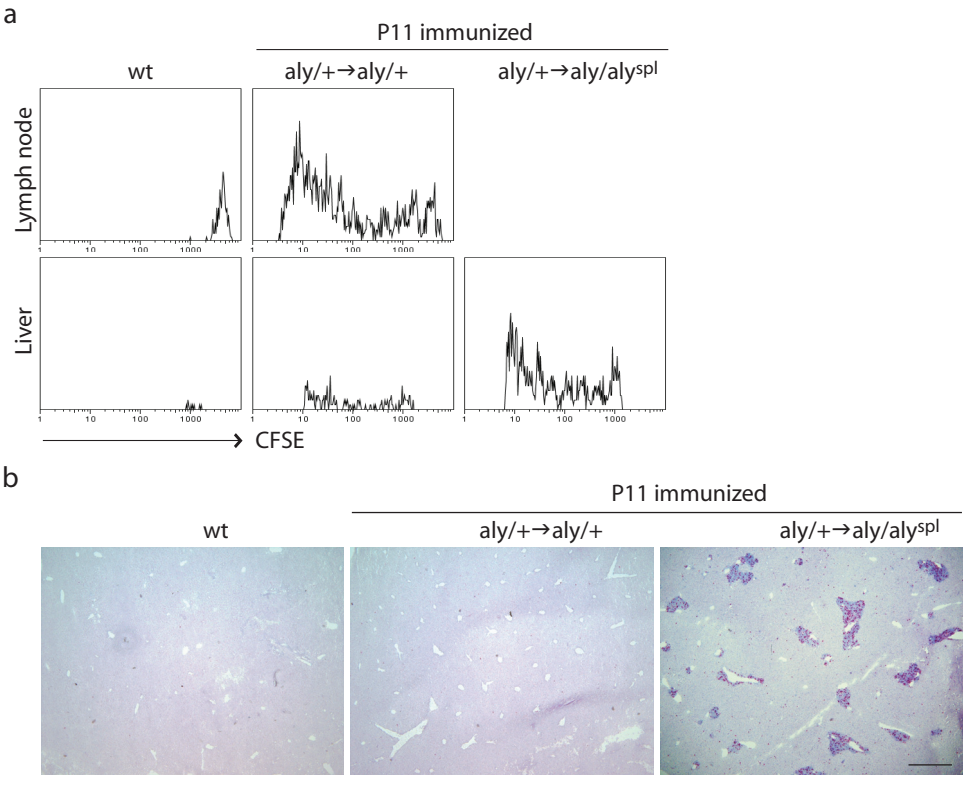
Figure 4



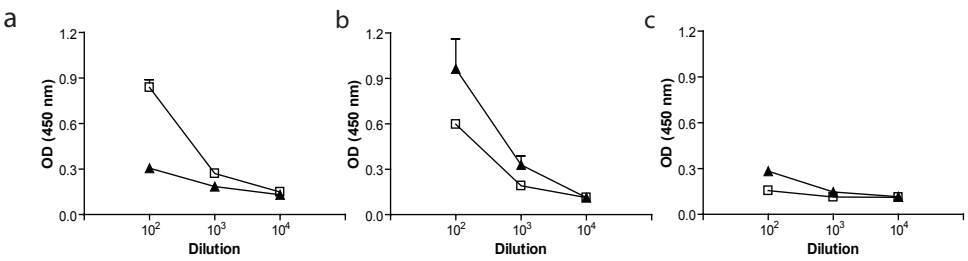
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3



nature medicine

Experimental autoimmune encephalomyelitis repressed by microglial paralysis

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Although microglial activation occurs in inflammatory, degenerative and neoplastic central nervous system (CNS) disorders, its role in pathogenesis is unclear. We studied this question by generating *CD11b-HSVTK* transgenic mice, which express herpes simplex thymidine kinase in macrophages and microglia. Ganciclovir treatment of organotypic brain slice cultures derived from *CD11b-HSVTK* mice abolished microglial release of nitrite, proinflammatory cytokines and chemokines. Systemic ganciclovir administration to *CD11b-HSVTK* mice elicited hematopoietic toxicity, which was prevented by transfer of wild-type bone marrow. In bone marrow chimeras, ganciclovir blocked microglial activation in the facial nucleus upon axotomy and repressed the development of experimental autoimmune encephalomyelitis. We conclude that microglial paralysis inhibits the development and maintenance of inflammatory CNS lesions. The microglial compartment thus provides a potential therapeutic target in inflammatory CNS disorders. These results validate *CD11b-HSVTK* mice as a tool to study the impact of microglial activation on CNS diseases *in vivo*.

Microglial cells are of hematopoietic origin and populate the CNS early during development to form a regularly spaced network of ramified cells[1]. Microglia become rapidly activated in most pathological conditions of the CNS. Although microglial activation has been described extensively in many CNS diseases[1], its impact on disease pathogenesis remains ill defined[1,2]. In autoimmune diseases such as multiple sclerosis, most data point to a detrimental role of microglia, for example by producing neurotoxic molecules, proinflammatory cytokines, chemokines or by presenting self-antigens[3–6]. But there have been claims that microglial activation in other diseases may counteract the pathogenic changes by providing neurotrophic or immunosuppressive factors[7,8].

We wished to investigate the role of activated microglia using a pharmacogenetically inducible *in vivo* model of microglial paralysis. We have therefore generated transgenic mice in which the thymidine kinase of herpes simplex virus (encoded by *HSVTK*) is driven by the *CD11b* promoter[9]. CD11b, encoded by *Itgam*, is the alpha chain of the Mac-1 integrin and is expressed in cells of myeloid origin, including macrophages and microglia. We used a 1.7-kilobase *CD11b* promoter fragment which drives sustained expression in macrophages of transgenic mice at levels similar to those of the endogenous *CD11b* gene[9]. *HSVTK* is a suicide gene that converts antiviral nucleotide analog prodrugs such as ganciclovir (GCV) to a monophosphorylated form, which is then transformed into a toxic triphosphate by endogenous cellular kinases[10]. Expression of HSVTK renders preferentially proliferating cells sensitive to GCV, as the active metabolite competes with thymine for DNA synthesis. This strategy has been used to selectively ablate defined cell lineages, for example in transgenic mice[11,12].

Nondividing HSVTK$^+$ cells also show susceptibility to GCV, albeit at reduced levels: in this case, toxicity has been ascribed to interference with mitochondrial DNA synthesis[13]. Here we report that microglial cells of *CD11b-HSVTK* transgenic mice, although mostly resting in the adult CNS, are efficiently paralyzed by GCV administration. We took advantage of this phenomenon to study the contribution of microglial activation to two disease models: facial nerve transection and experimental autoimmune encephalitis.

RESULTS

Characterization of transgenic mice

Before generating transgenic mice, the *CD11b-HSVTK* transgene was stably transfected into the BV-2 microglial cell line, and GCV was added to the culture medium. We detected efficient, dose-dependent killing of microglial cells with a significant difference between BV2-TK and controls at 2, 10 and 20 μm GCV ($P < 0.001$, one-way ANOVA test), confirming the functionality of the suicide approach (**Fig. 1a**). We then established two independent transgenic mouse lines denoted B6,D2-Tg(CD11b-HSVTK)618Zbz (*tg*618) and B6,D2-Tg (CD11b-HSVTK)620Zbz (*tg*620), and confirmed integration of the transgene by

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Southern blotting and PCR of genomic DNA (**Fig. 1b**,**c**). In the absence of GCV, transgenic offspring showed no phenotypic alterations except for male sterility, which is a known consequence of ectopic HSVTK expression[14]. Line *tg*620 was backcrossed to C57BL/6 mice for nine generations and was used for all of the experiments described below.

Expression of HSVTK was ascertained by western blot analysis of various organs. As expected, all macrophage-containing organs, including spleen, lung and brain, showed sustained expression of the *HSVTK* transgene (**Fig. 1d**,**e**). Western blot analysis of purified microglial and astroglial cells derived from *CD11b-HSVTK* mice established that transgene expression within the CNS was present in microglial cells but not in astrocytes (**Fig. 1f**). Addition of GCV to primary microglial cell cultures (>98% purity, **Fig. 1f**), or to mixed glial cell cultures, led to lineage-specific death of microglial cells (**Fig. 1g**,**h**).

We then administered GCV to *tg*620 mice intraperitoneally or in drinking water. Circulating CD11b$^+$ cells were substantially reduced after 3 d and almost entirely ablated after 5–6 d, whereas B- and T-cell counts were essentially unaltered (**Fig. 2a**). After 7 d of GCV treatment, *tg*620 mice developed fatal aplastic anemia with reduced erythroid and myeloid cell compartments in both bone marrow and peripheral blood (**Supplementary Fig. 1** and **Supplementary Table 1** online). Analysis of prenatal hematopoiesis in *tg*620 embryos at embryonic day 14.5 showed GCV-mediated ablation of CD11b$^+$ AA4.1$^+$ hematopoietic precursor cells[15] (**Supplementary Fig. 1** online), which therefore seem to be crucial for normal hematopoietic development.

Microglia-restricted HSVTK expression in *tg*620^{chi} mice

To restrict *HSVTK* expression to microglial cells and to overcome GCV-mediated myelotoxicity, we generated bone marrow chimeras. For donors, we used fetal liver cells (FLCs) or bone marrow derived from wild-type mice or congenic C57BL/6-β-actin-GFP mice[16]. Lethally irradiated *tg*620 transgenic mice were used as recipients. This strategy is based on the fact that monocytes and extraneural tissue macrophages are rapidly and efficiently repopulated upon adoptive bone marrow transfer, whereas recruitment of radioresistant microglia from the peripheral hematopoietic pool to the CNS is slow and rather inefficient[17–20]. Chimeric *tg*620 mice hosting wild-type bone marrow (termed *tg*620^{chi}) showed stable numbers of peripheral CD11b$^+$ monocytes and macrophages upon long-term GCV treatment (**Fig. 2b**). In reciprocal experiments, *tg*620 bone marrow was transferred to wild-type recipients. The latter mice experienced GCV-mediated loss of peripheral CD11b$^+$ cells and aplastic anemia (**Fig. 2b**) similar to *tg*620 mice. Intactness of the blood-brain barrier in both GCV-treated and untreated *tg*620^{chi} mice was shown by the lack of increased IgG influx into the CNS[21]. This excluded the possibility of radiation-induced blood-brain barrier leakage (**Supplementary Fig. 2** online).

Microglial paralysis after GCV treatment

To assess the efficiency and specificity of paralyzing microglia in *tg*620^{chi} mice, we performed unilateral transections of the facial nerve.

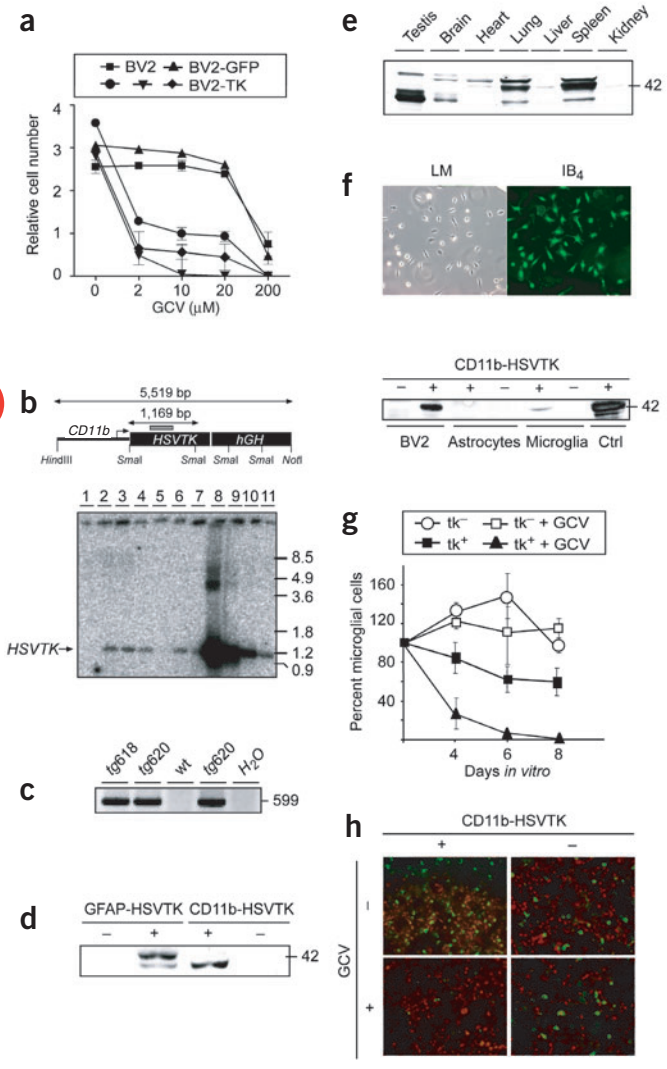

Figure 1 Characterization of *CD11b-HSVTK* transgene and transgenic mice. (**a**) Cell viability assay of microglial BV-2 cell clones stably transfected with *CD11b-HSVTK* (BV2-TK; circles, inverted triangles and diamonds). CMV-GFP-transfected BV-2 (BV2-GFP; triangles) and untransfected BV-2 controls (BV2; squares) served as negative controls. Ordinate: mean and s.d. of cell numbers on day 6 divided by cell numbers on day 0. (**b**) *CD11b-HSVTK* transgene depicting the *CD11b* promoter, the *HSVTK* gene and the human growth hormone gene (*hGH*) providing splice donor/acceptor sites and a polyadenylation signal[9]. For the Southern blot (below), an *HSVTK*-specific 530-bp PCR probe (hatched box) was hybridized to a fragment of 1169 bp (arrow on the left) in transgene-positive *tg*618 (lane 2: founder; lane 3: F1 offspring) and *tg*620 mice (lane 4: founder; lane 6 and 7: F1 offspring) and not in negative littermates (lanes 1,5). As positive control, 10-fold dilutions of *CD11b-HSVTK* plasmid were spiked in genomic DNA of wild-type mice (ranging from 1,125 pg (lane 8) to 1.125 pg (lane 11)). Larger bands in lanes 8 and 9 correspond to incompletely digested plasmid. Right, molecular weight in kb. (**c**) PCR of genomic DNA resulted in a transgene-specific band at 599 bp. (**d**) Western blot of brain tissue: *HSVTK* transgene is expressed in CD11b-HSVTK (lane 3) and GFAP-HSVTK brains[12] (lane 2, positive control). No signal in negative littermates (lanes 1, 4). Right, molecular weight in kDa. (**e**) Western blot of various organs: strong expression of HSVTK in testis[14] (lane 1) and in macrophage-containing organs. Up to four HSVTK proteins were detectable due to cryptic translational initiation sites in the coding region of *HSVTK*[46]. (**f**) Western blot of isolectin IB$_4^+$–microglial (upper right, green color; left, light microscopic (LM) image) and astrocyte cell culture lysates of *tg*620 mice shows HSVTK protein expression in microglial cells (lane 5), but not in astrocytes (lane 3). *Tg*620 spleen homogenate (lane 7) and stably *CD11b-HSVTK*-transfected microglial BV-2 cells (lane 2), positive controls; microglia and astrocytes of non-transgenic littermates (lane 6 and 4) and GFP-transfected BV-2 cells (lane 1), negative controls. (**g**) Strong reduction of transgenic microglial cells in the presence (filled triangle), but not in the absence of GCV (filled squares), whereas wild-type microglia in the presence (open squares) or absence (open circles) of GCV were stable. Number of microglia was normalized to 100% at day 0 and experiments were done in triplicates. (**h**) Whereas microglia (IB$_4^+$, green) were strongly reduced in mixed glial cell cultures derived from *tg*620 mice, GFAP$^+$ astrocytes (red) appeared to be unaffected by GCV (lower left).

Subsequent retrograde degeneration strongly activates microglia within the respective facial motor nucleus[22]. Here, GCV administration to *tg*620^{chi} mice considerably repressed activation of microglia (**Fig. 3a**), most likely as a result of activation-induced upregulation of *CD11b-HSVTK* which hypersensitizes microglia to GCV. No differences were seen in microglial activation within lesioned facial nuclei of non-GCV-treated *tg*620 mice or nontransgenic littermates in the presence or absence of GCV (data not shown). GCV-mediated microglial paralysis of *tg*620^{chi} mice for 7 weeks in the absence of a pathological stimulus did not result in obvious phenotypic or histopathological changes except for a decrease in microglial cell numbers as assessed by FACS analysis (**Supplementary Fig. 3** online). Although it is theoretically possible that ablation of microglia in nondiseased chimeric *tg*620 mice may result from radiation toxicity, we consider this unlikely, as others have found normal numbers of ramified, resting microglia after irradiation at time points similar to the ones described here[19].

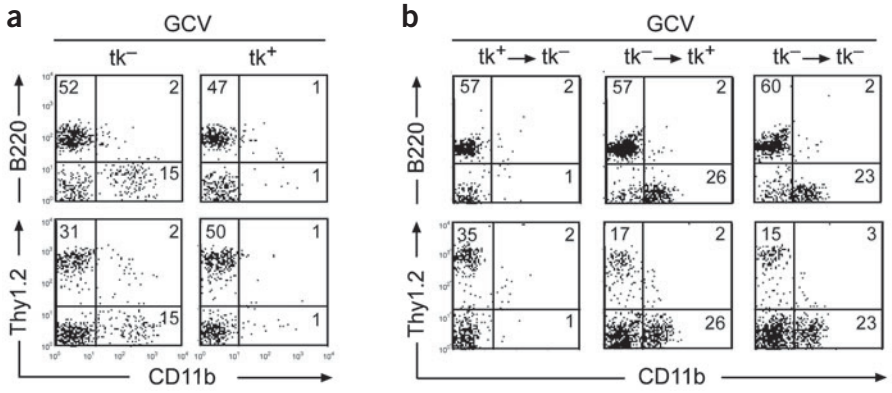

Figure 2 *In vivo* characterization of *tg*620 mice and FACS analysis of peripheral blood. (**a**) In *tg*620 (tk$^+$) mice, we observed a drastic GCV-mediated decrease of CD11b$^+$ monocytes and granulocytes, whereas B220$^+$ B cells and Thy1.2$^+$ T cells were unaffected (right panels). GCV treatment of nontransgenic littermates (tk$^-$), even at doses that were 10 times higher, did not elicit alterations in the blood leukocyte numbers (left panels). (**b**) In *tg*620^{chi} mice, the GCV-mediated loss of CD11b$^+$ cells was rescued upon adoptive transfer of wild-type bone marrow (tk$^-$ → tk$^+$; middle panels). *Tg*620^{chi} mice were indistinguishable from nontransgenic littermates receiving the same treatment (tk$^-$ → tk$^-$; right panels). Adoptive transfer of *tg*620 bone marrow into wild-type mice (tk$^+$ → tk$^-$; left panels) restored susceptibility of CD11b$^+$ cells to GCV. Thy1.2$^+$ T cells and B220$^+$ B cells were unaffected in all experimental groups. Numbers indicate percentage of cells. Arrows indicate transfer of bone marrow.

We then assessed the functional consequences of microglial paralysis in organotypic hippocampal slice cultures (OHSCs). OHSCs derived from *tg*620 mice were stimulated with lipopolysaccharide (LPS) and interferon (IFN)-γ, and microglial activation in the presence or absence of GCV was determined. GCV treatment of stimulated OHSCs inhibited microglial activation (**Supplementary Fig. 4** online) and abrogated the release of nitrite, tumor necrosis factor (TNF) and macrophage inflammatory protein (MIP)-1β (**Fig. 3b–d**). These factors are thought to interfere with CNS homeostasis either by damaging tissue directly, or by attracting and/or activating other immune cells including autoreactive T cells in experimental autoimmune encephalitis (EAE)[5].

Repression of EAE in bone marrow chimeric *tg*620 mice

Having established that GCV treatment of *tg*620^{chi} mice resulted in profound paralysis of microglia within the CNS, we studied the impact of microglial paralysis on the pathogenesis of EAE. Age-matched female *tg*620^{chi} and wild-type mice were immunized with an encephalitogenic synthetic myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) peptide. Untreated *tg*620^{chi} mice and wild-type mice reconstituted with wild-

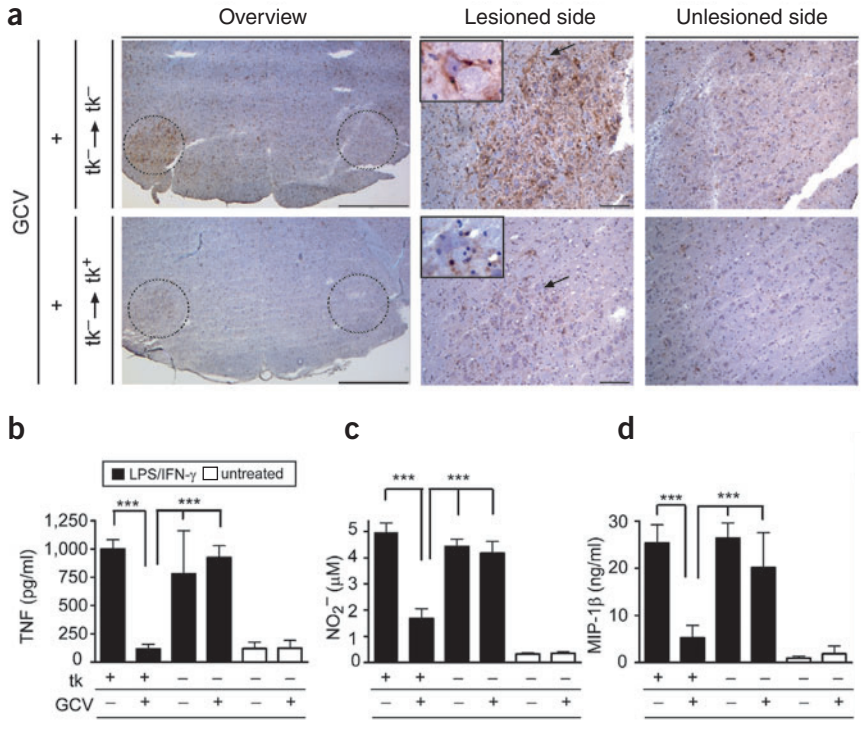

Figure 3 Microglial paralysis in *tg*620 mice. (**a**) GCV-mediated microglial paralysis within the facial nucleus of *tg*620^{chi} mice 7 d after axotomy of the left facial nerve. Strong activation and proliferation of CD11b$^+$ microglial cells (brown) within the corresponding facial motor nucleus of GCV-treated bone marrow chimeric wild-type mice (tk$^-$ → tk$^-$; upper left and middle) was seen versus complete inhibition of microglial activation within lesioned facial nuclei of GCV-treated *tg*620^{chi} mice (tk$^-$ → tk$^+$; lower left and middle). Activated (insert, upper middle panel) but not paralyzed microglia (insert, lower middle panel) was attached to cell bodies of injured neurons. $n = 3–4$ mice/group. Circles indicate the facial motor nuclei. Scale bars: left panel, 1 mm; middle and right panels, 100 μm. (**b–d**) GCV treatment abrogated the LPS-IFN-γ-induced (black bars) release of microglial TNF (**b**), nitrite (**c**) and MIP-1β (**d**) in OHSCs from *tg*620 mice. White bars, nonstimulated OHSCs. Statistical significance was assessed by a one-way ANOVA test. ***$P < 0.001$.

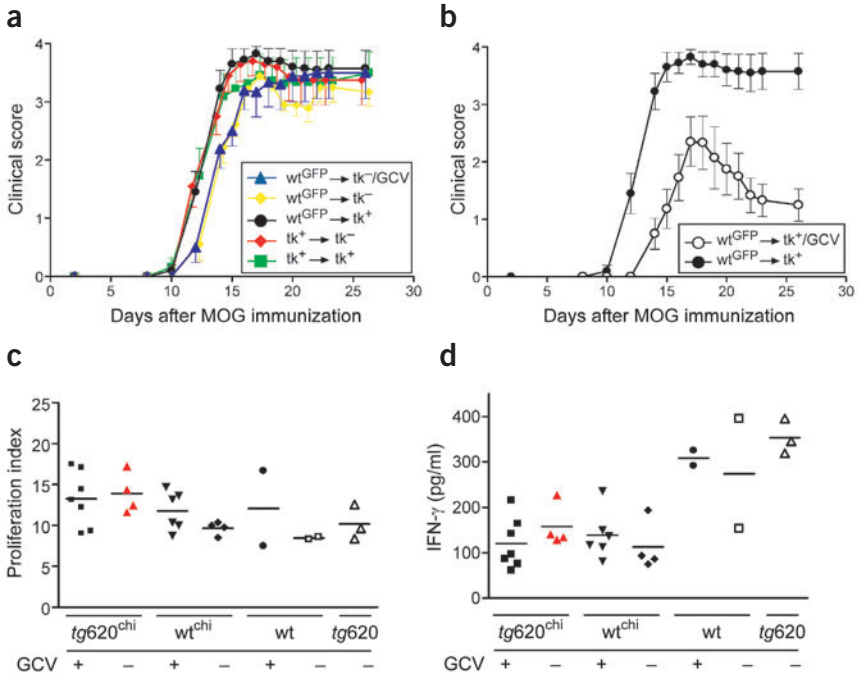

Figure 4 Microglial paralysis represses clinical EAE in *tg*620^{chi} mice. (**a**,**b**) Clinical EAE score of bone marrow chimeric mice upon MOG immunization: all control mice showed severe EAE signs (**a**). GCV-treated bone marrow chimeric *tg*620^{chi} mice showed considerably repressed EAE (open circles). Omission of GCV treatment of bone marrow chimeric *tg*620^{chi} mice resulted in severe EAE (closed circles) (**b**). Controls consisted of congenic β-actin-GFP (wt^{GFP}) bone marrow chimeric wild-type mice (tk^{-}) treated (wt^{GFP} → tk^{-}/GCV, blue triangle) or not treated (wt^{GFP} → tk^{-}, yellow diamond) with GCV, *tg*620^{chi} mice (wt^{GFP} → tk^{+}, black circle) and wild-type mice with transgenic *tg*620 bone marrow (tk^{+} → tk^{-}, red diamond) and *tg*620 mice with transgenic *tg*620 bone marrow (tk^{+} → tk^{+}, green square), the latter two in the absence of GCV. Arrows indicate bone marrow transfer. Experiments were repeated three times for bone marrow chimeric *tg*620 and wild-type mice with or without GCV and showed identical results. n = 9–13 mice/group. (**c**,**d**) GCV-treated *tg*620^{chi} mice are fully capable of driving T_H1 immunity. Recall proliferation (**c**) and IFN-γ secretion (**d**) of encephalitogenic T cells in response to MOG in the presence or absence of GCV. Experimental groups consisted of *tg*620^{chi} (filled black squares and filled red triangles), wt^{chi} (filled black triangles and filled black diamonds), wild-type (filled black circles and open squares) and *tg*620 mice (open triangles) treated with GCV or not, as depicted. Each symbol represents the mean for triplicate cultures; bars indicate the mean proliferation per experimental group. The proliferation index is calculated as fold increase in T-cell proliferation upon MOG recall compared to medium only. A detailed analysis of *tg*620^{chi} T-cell proliferation upon MOG recall is shown in **Supplementary Fig. 6** online.

type bone marrow showed similar onset and severity of EAE (**Fig. 4a**), whereas GCV treatment of *tg*620^{chi} mice resulted in a considerable delay in disease onset and in repression of clinical EAE signs (**Fig. 4b**; $P < 0.01$–0.05, one-way ANOVA test including Bonferroni's Multiple Comparison post test). Conversely, comparison of transgenic *tg*620 mice with nontransgenic littermates (with or without GCV) showed indistinguishable clinical disease development (**Supplementary Fig. 5** online). GCV itself did not influence EAE, as the clinical course of bone marrow–reconstituted wild-type littermates of *tg*620 mice was identical to that of wild-type bone marrow–reconstituted mice regardless of GCV treatment (**Fig. 4a**).

Notably, GCV administration did not alter the cellular composition and function of the peripheral immune system of *tg*620^{chi} mice. FACS analysis of peripheral blood showed no abnormalities in relative numbers of Thy1.2^{+} T cells, B220^{+} B cells, as well as CD11b^{+} monocytes and granulocytes (data not shown). GCV treatment of *tg*620^{chi} and control mice also did not alter the proliferative response of T cells from mice immunized with keyhole limpet hemocyanin (KLH; data not shown) or MOG in *in vitro* recall assays (**Fig. 4c** and **Supplementary Fig. 6** online). In addition, levels of IFN-γ and interleukin (IL)-2 were similar in GCV-treated versus untreated *tg*620^{chi} mice after recall with KLH (data not shown) or MOG ($P > 0.05$, one-way ANOVA and Tukey post test), except for a difference in IFN-γ production between nonchimeric and chimeric mice, which can be attributed to the reconstitution procedure (**Fig. 4d**). MOG recall assays allowed us to directly address the performance of encephalitogenic T cells in response to their cognate antigen upon *in vivo* priming. The results suggest that T cells of GCV-treated chimeric *tg*620 mice are fully capable of inducing expansion and effector function of MOG-reactive lymphocytes compared to their non-GCV-treated counterparts (**Fig. 4c**,**d** and **Supplementary Fig. 6** online). These experiments validate that GCV treatment does not alter a number of critical immune functions of *tg*620^{chi} mice, including the ability to mount an encephalitogenic immune response.

Histological examination showed that clinical EAE signs correlated with the extent of inflammatory CNS infiltrates (**Fig. 5a–c**). Immunohistochemical staining for Iba1, which identifies monocytes, macrophages and microglia[23], showed positive infiltrates mainly within the spinal and cerebellar white matter (**Fig. 5a**,**b**). Accordingly, both myelin and axons seemed to be damaged (**Fig. 5a**). But, in line with the clinical performance, MOG-immunized *tg*620^{chi} mice treated with GCV showed few Iba1^{+} infiltrates and did not show major myelin or axonal destruction (**Fig. 5a**). We detected very little activated Iba1^{+} microglia in the CNS of GCV-treated *tg*620^{chi} mice, whereas all control mice showed strongly activated Iba1^{+} microglial cells (**Fig. 5a**,**b**). MOG-immunized bone marrow chimeric wild-type control animals, in the presence or absence of GCV, regularly showed strong inflammatory changes (data not shown) indistinguishable from non-GCV-treated *tg*620^{chi} mice.

We then quantified and characterized inflammatory white-matter infiltrates by FACS analysis of mononucleated cells derived from spinal cord and brain stem tissue 14 d after MOG immunization (*i.e.*, at the peak of clinical signs). In line with the histological findings, we found a high percentage of infiltrating cells in CNS tissues of non-GCV-treated *tg*620^{chi} mice, which consisted of CD45^{high} CD11b^{-} lymphocytes as well as of CD45^{high} CD11b^{+} myeloid cells. Further analysis showed that approximately two-thirds of the CD45^{high} CD11b^{-} infiltrating lymphocytes were CD4^{+} cells, whereas only a minority consisted of CD8^{+} cells (**Fig. 5c**). GCV treatment of *tg*620^{chi} mice substantially reduced inflammatory infiltrates: lymphocytes were decreased from 36% to 10%, whereas the amount of infiltrating CD45^{high} CD11b^{+} monocytes was reduced by 40% (**Fig. 5c**). As expected, there were no obvious differences in the numbers of CD45^{intermediate} CD11b^{+} microglial cells in GCV-treated versus untreated *tg*620^{chi} mice. Moreover, the numbers of GFP^{+} microglia recruited from the extraneural pool

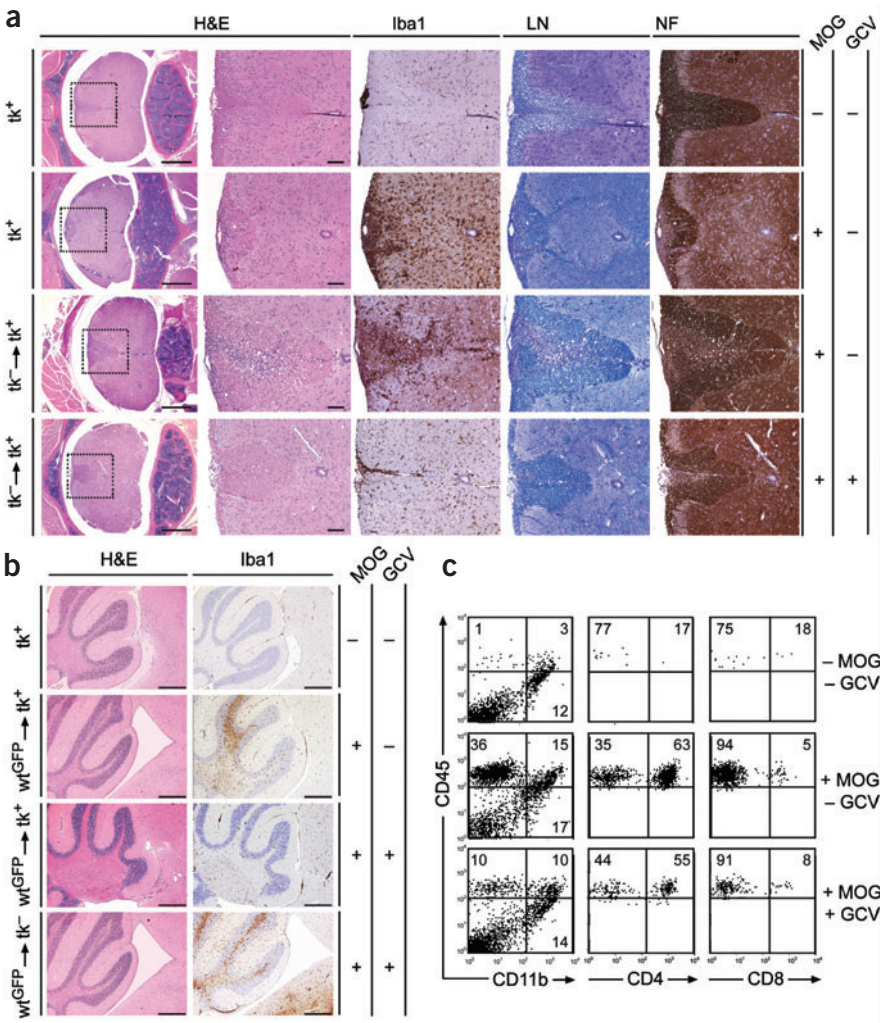

Figure 5 Microglial paralysis represses EAE-associated inflammatory infiltrates in *tg*620^{chi} mice. (**a**) Spinal cords of MOG-immunized and control mice (H&E, hematoxylin and eosin). Inflammation was largely confined to the white matter, leading to destruction of myelin tracts (LN, Luxol-Nissl stain) as well as axonal integrity (NF, neurofilament). No inflammation was seen in *tg*620 mice in the absence of MOG immunization (upper panel). Scale bar, 200 μm except for left column, 500 μm. (**b**) Cerebella of MOG-immunized mice: *tg*620^{chi} mice (wt^{GFP} → tk^{+}) in the absence of GCV (second panel from top) or of wt^{GFP} bone marrow chimeric wild-type animals (wt^{GFP} → tk^{-}) treated with GCV (lower panel) or not (data not shown) showed severe inflammation mainly confined to the white matter. But *tg*620^{chi} mice treated with GCV (third panel from top) showed neither activated Iba1^{+} microglia nor infiltrating leucocytes. No inflammation in *tg*620 mice in the absence of MOG-immunization (upper panel). Scale bar, 500 μm. (**c**) FACS analysis of spinal cord and brain tissue 14 d after MOG immunization. Whereas non-MOG-immunized *tg*620^{chi} mice without (upper row) or with GCV (data not shown) showed few CD45^{high} CD11b^{-} lymphocytes in the CNS, MOG immunization induced a strong inflammatory infiltration in *tg*620^{chi} mice in the absence of GCV (middle row) consisting of CD45^{high} CD11b^{-} lymphocytes and CD45^{high} CD11b^{+} monocytes. GCV-mediated microglial paralysis resulted in a substantial reduction in inflammatory infiltrates in *tg*620^{chi} mice (lower row). Infiltrating CD45^{high} CD11b^{-} lymphocytes consisted mainly of CD4^{+} T cells and few CD8^{+} T cells (middle and right). Numbers indicate percentages of cells. Each experimental group consisted of pooled homogenate derived from three mice representing the average clinical EAE score.

of macrophages in MOG-immunized mice were similar in the absence or presence of GCV (**Supplementary Fig. 7** online).

DISCUSSION

The data described here indicate that GCV administration to *tg*620 mice brings about efficient conditional paralysis of microglia *in vivo*. Treatment with GCV *in vivo* results in substantial inhibition of microglial activation upon facial axotomy, and abolishes the release of microglia-derived nitrite and proinflammatory cytokines and chemokines from activated brain-slice cultures. In EAE, microglial paralysis results in substantial amelioration of the clinical signs and in strong reduction of CNS inflammation. As facial nerve transection causes no disruption of the blood-brain barrier[22], we conclude that GCV diffuses adequately into the intact CNS.

Immunomodulatory compounds, including IFN-β, copolymer I and statins, can repress CNS autoimmune diseases including EAE[24–26]. But these drugs exert additional ill-defined effects on many immune cells, including T cells and peripheral monocytes. Inhibition of peripheral macrophages has been shown to prevent EAE[27,28], whereas microglial activation is thought to be secondary to infiltration of lymphocytes[2]. Accordingly, the pathogenesis of EAE is widely held to be triggered exclusively within the peripheral immune system[2,29].

Whereas the importance of autoreactive CD4^{+} T cells in EAE has been extensively documented, particularly by their ability to initiate disease, the effector mechanism leading to inflammation and demyelination within the CNS is likely to be provided by other cell types such as infiltrating macrophages and resident microglia[29]. But as a result of the dearth of suitable animal models, a definitive dissection of potential effector cell types involved, namely infiltrating macrophages and resident microglia, has proven difficult. Early studies pointing to a more direct role of CNS-derived cells in the pathogenesis of EAE included the repression of EAE in bone marrow chimeric mice lacking CD40 or IL-23 predominantly in the CNS[30,31]. But these studies did not exclude other CNS residents than microglia as a potential source of these immunomodulators.

What are the mechanisms of microglia-mediated damage in EAE? Nitric oxide and its adducts may disrupt CNS tissue integrity[26,32], whereas microglia-derived cytokines and chemokines such as TNF and MIP-1β activate and attract blood-derived leukocytes. These may in turn interfere with CNS homeostasis (*e.g.*, by damaging myelin)[5,26]. Reactivation of myelin-specific T cells within the CNS upon recognition of local autoantigens is critical to induce and/or sustain EAE[33]. But it is still debated whether microglia present myelin-associated antigens to autoreactive T cells *in vivo*: it has been reported that the antigen-presenting capacity of CD45^{intermediate} CD11b^{+} microglia is not essential for promoting encephalitogenic myelin-specific CD4^{+} T cells *in vitro*[34], whereas others regard microglial presentation of myelin-associated antigens as critical[6,35]. Our data clearly establish

that microglia are crucial for the development of EAE, which seems to be conferred by the release of cytokines and chemokines as well as reactive oxygen species. This view is in agreement with previous reports showing that chemokine secretion by CNS residents *in vivo* is vital for disease development[30].

Even in the late neurodegenerative phase of EAE, which goes along with destruction of neurons, activated microglia may have a crucial role. Recent reports suggest an inverse relationship between microglial activation and neurogenesis[36–38]. One might therefore speculate that reduced neurogenesis, by decreasing the capacity for neural recovery, may exacerbate EAE.

From a translational viewpoint, the results reported here suggest that microglial activation may represent a target in the therapy of multiple sclerosis and other immune-mediated CNS diseases. In addition to providing insights into the pathogenesis of autoimmune CNS diseases, *tg*620 mice are proving a useful tool for the study of microglial involvement in a broad variety of neuroimmune and neurodegenerative diseases, including encephalitides, Alzheimer, Parkinson, prion and motor neuron diseases.

METHODS

Transgenic mice. A 544-base pair *Bam*HI fragment containing the *Thy1* cDNA in pB203 derived from the *CD11b-Thy1-human growth hormone (GH)* cDNA[9] (provided by D.G. Tenen) was replaced by the *Bgl*II to *Bam*HI *HSVTK* fragment[39]. Correct construction of the transgene was confirmed by sequencing and by assessing GCV-mediated cell death of BV-2 microglial cells stably transfected with *CD11b-HSVTK* using electroporation in accordance with standard protocols. Following *Not*I and *Hind*III excision, we introduced *CD11b-HSVTK* into fertilized B6D2F2 hybrid eggs by pronuclear microinjection. We analyzed genomic integration by Southern blotting of *Sma*I-digested genomic DNA according to standard procedures. An *HSVTK*-specific 530-bp PCR probe was generated and labeled with $^{\alpha 32}$P dCTP. The following primers were used (Microsynth): 5′-ACAATGGGCATGCCTTATGC-3′ and 5′-GGACATATTGCACAAACGGA-3′. For routine genotyping, we performed PCR analysis on tail DNA in line with standard protocols using the following primers (Microsynth): 5′-GACTTCCGTGGCTTCTTGCTGC-3′ and 5′-GTGCTGGCATTACAGGCGTGAG-3′.

Bone marrow chimeric mice and GCV administration. We generated bone marrow chimeric mice as described[30] with bone marrow of adult mice or FLCs, embryonic day 13.5–14.5. Recipient mice were lethally irradiated with 950 rad and injected intravenously either with 2×10^7 bone marrow cells or 1×10^7 FLCs. Engraftment took place over 6–8 weeks. We defined successful reconstitution as >95% engraftment of blood leukocytes by FACS analysis. Congenic C57BL/6 β-actin-GFP donor mice[16] (Jackson Laboratories) were bred in house under pathogen-free conditions. We achieved GCV administration by intraperitoneal injection of 100 μg GCV (Cymevene; Roche) per gram of body mass every 2 d or orally by adding 60 μg/ml GCV to the drinking water.

Western blot analysis. We prepared 10% (wt/vol) homogenates of various organs or cell cultures according to standard procedures[40]. We used a polyclonal rabbit serum to HSVTK[41] (1:5,000; provided by W.C. Summers) followed by incubation with a rabbit IgG-HRP-specific antibody (1:2,500; Zymed). Equal loading of protein (50 μg/lane) was assured by a bicinchoninic acid (BCA) assay according to standard procedures.

Cell cultures. Cultures of purified microglia and astrocytes were prepared and maintained as described[42,43] in the presence or absence of 2.5 μg/ml GCV. Four high-power fields of three wells were counted on days 0, 3 and 6 using an inverted microscope (Zeiss). We identified microglia by fluorescein isothiocyanate–labeled Isolectin-B4 (1:50; Sigma); astrocytes were stained with an antibody to glial fibrillary acidic protein (1:300; Dako) and visualized by an Alexa 546-labeled secondary rabbit IgG-specific antibody (1:300; Juro). We performed fluorescence microscopy on a Zeiss microscope (Axioplan 2) equipped with a digital camera (Axiocam).

Organotypic hippocampal slice cultures. OHSCs were prepared from 12-d-old mice as described[44], and, where indicated, treated with 5 μg/ml GCV from day 0 for the duration of the experiment. After 7 d, we initiated activation of microglia by adding IFN-γ (10 ng/ml) and LPS (1 μg/ml). To assess nitric oxide adducts, cytokines and chemokines, cell culture supernatant was harvested 48 h after LPS and IFN-γ stimulation except for TNF measurements, for which supernatant was taken after 8 h. Each group contained 3–4 inserts with 4 OHSCs on each insert.

Induction and evaluation of EAE. We subjected 13–16-week-old mice to subcutaneous administration of 200 μg of $MOG_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK; Neosystem) emulsified in complete Freund adjuvant supplemented with 4 mg/ml *Mycobacterium tuberculosis* (DIFCO), as described[30]. Mice received intraperitoneal injections with 200 ng pertussis toxin (Sigma) at the time of immunization and 48 h later. GCV administration started 7 d before MOG immunization. Mice were scored as described[31]. Animal experiments were approved by the Swiss Veterinary Office (#203/98, 40/2002, 85/2003 and 136/2004).

Histology. Whole mouse brains or spinal columns were fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin or snap-frozen in liquid nitrogen. Antibodies to glial fibrillary acidic protein (1:300; Dako), synaptophysin (1:50; Zymed), microtubule-associated protein-2 (1:1,000; Sigma), neurofilament protein (200-kDa subunits; 1:20; Bio-Science) and Iba1 (1:100; Wako Chemicals) were used. We used CD11b-specific antibodies (MCA 711, 1:1,000; Serotec) only on cryosections.

Flow cytometry. We removed spinal cords and processed them for FACS analysis as described[30,31]. Peripheral blood or spleen and FLC suspensions were analyzed according to standard protocols[40]. Fluorescein isothiocyanate–, phycoerythrin- or peridinin chlorophyll-a protein–conjugated monoclonal antibodies to mouse CD11b, B220, CD8, CD4, Thy1.2, AA4.1, CD45 or biotinylated CD8- or CD11c-specific antibodies were used, the latter two coupled to a secondary streptavidin-labeled allophycocyanin antibody (Becton Dickinson). Data were acquired on a FACScalibur (Becton Dickinson).

Recall responses. Mice were primed by flank injections of 200 μg KLH (Sigma) or 200 μg $MOG_{35–55}$ peptide (Neosystem) emulsified in complete Freund adjuvant. Mice received intraperitoneal injections of 200 ng pertussis toxin (Sigma) at the time of immunization. After 7 d, the axillary and inguinal lymph nodes were removed and homogenized. We placed 2×10^5 lymph node cells as triplicates in a 96-well plate and pulsed them with 100 μg KLH or MOG or 10 μg ConA (Sigma). After 24 h, cells were pulsed with ^{3}[H]-thymidine (NEN-DuPont; final concentration 5 μCi/ml) and incubated for an additional 24 h before they were harvested and thymidine incorporation was assessed using a Filtermate harvester (Packard Meriden) and TopCount-NXT Packard Microplate scintillation and luminescence counter. Supernatants of sister cultures were harvested after 48 h and analyzed by ELISA.

Enzyme-linked immunosorbent assay (ELISA) and nitrite measurement. Supernatants derived from recall assays or OHSCs were analyzed by the use of ELISA kits for TNF, IFN-γ, IL-2 (Pharmingen) or MIP-1β (R&D systems) according to the manufacturer's instructions. Nitrite was measured with the Griess reagent. We mixed 50 μl supernatant or $NaNO_2$ standards with 25 μl *N*-(1-naphthyl)ethylenediamine (0.1% in water) and 25 μl sulfanilamide (1% in 1.2 N HCl) in a 96-well plate and the optical density was assessed after 3 min at 570–690 nm. To include the contribution of the NO metabolite nitrate, we added 50 μl vanadium(III) chloride (8 mg/ml in 1M HCL) to the Griess reagent and incubated it at 37 °C for 30 min[45].

Facial nerve axotomy. We anesthetized 13–16-week-old mice with ketamin/rompun according to published protocols. The left facial nerve was transected at the stylomastoid foramen, and the animals were killed using CO_2 after a survival time of 7 d[22]. Successful axotomy was assumed on the basis of immobile whiskers on the lesioned side. GCV administration started 1 week before axotomy. Facial nerve axotomy experiments were approved by the UK Home Office (PPL 70/5341 to G.R.).

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis

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Immunization with myelin antigens leads to the development of experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. The disease can also be induced by the transfer of encephalitogenic CD4$^+$ T helper (T_H) lymphocytes into naive mice. These T cells need to re-encounter their cognate antigen in the context of major histocompatibility complex (MHC) class II–bearing antigen-presenting cells (APCs) in order to recognize their target. The cell type and location of the APC mediating T-cell entry into the central nervous system (CNS) remain unknown. Here, we show that APCs of the lymphoreticular system and of the CNS parenchyma are dispensable for the immune invasion of the CNS. We also describe that a discrete population of vessel-associated dendritic cells (DCs) is present in human brain tissue. In mice, CD11c$^+$ DCs alone are sufficient to present antigen *in vivo* to primed myelin-reactive T cells in order to mediate CNS inflammation and clinical disease development.

Multiple sclerosis is the most common inflammatory disease of the CNS, which is characterized by perivascular inflammatory lesions, demyelination and axonal damage[1]. Experimental autoimmune encephalomyelitis (EAE) serves as the animal model of multiple sclerosis and can be induced in susceptible rodent strains by active immunization with myelin antigens. During EAE, auto-aggressive myelin-reactive T lymphocytes migrate into the CNS, where they recognize their cognate target antigen and initiate an inflammatory cascade leading to tissue damage. The disease can also be induced by adoptively transferring an expanded population of myelin-reactive encephalitogenic CD4$^+$ T_H cells. Although the molecular requirements for naive T-cell priming and the formation of an immunologic synapse between a naive T cell and a competent APC are well characterized[2], little is known about the APC requirements of fully primed, activated T_H cells. Because even activated and expanded T cells cannot recognize native proteins in the absence of APCs, encephalitogenic T_H cells that enter the CNS need to re-encounter their cognate APCs in order to identify their target[3]. Oligodendrocytes are myelinating cells of the CNS and are thought to be the main target of such an antimyelin immune response. But oligodendrocytes do not express MHC class II molecules and can therefore not serve as APCs[4,5]. It is commonly believed that CNS-resident microglia or astroglial cells serve as local APCs to permit target recognition for invading lymphocytes[6,7]. This assumption is mainly based on *in vitro* studies and the identity of the CNS-resident APCs that are crucial for T-cell reactivation and disease onset and progression *in vivo* is a subject of intense debate[6,8–10].

Upon adoptive transfer of activated myelin-reactive T cells into recipient animals, there is a lag phase of several days before their invasion of the CNS and the development of clinical disease[11,12]. This lag phase has been interpreted to represent a crucial time period during which disease-causing T cells are reactivated. Attempting to show the crucial antigen-presenting events that occur during this lag phase, researchers have performed trafficking studies of myelin-reactive T cells. It was shown that myelin basic protein–reactive lymphocytes transferred into rats first enter secondary lymphoid tissues, where the expression of activation markers and chemokine receptors is synchronized before CNS entry[11]. Although these studies suggested a causal involvement of the peripheral immune system in inducing encephalitogenic activity of adoptively transferred effector T cells, an essential role of the systemic immune compartment in CNS inflammation and disease development was not established. Here we show that encephalitogenic T cells migrate into the CNS, recognize their target antigen and cause disease even in the absence of a lymphoreticular system. Furthermore, MHC class II–bearing cells within the CNS parenchyma, such as microglia, are not required to serve as APCs permitting lymphocyte entry and disease development. Using transgenic mice in which the APC capacity is restricted to DCs, we establish that a population of CD11c$^+$ (encoded by *Itgax*) DCs is sufficient to present antigen to autoreactive T cells in order to mediate CNS inflammation and clinical disease development. The presence of CD209 (DC-SIGN)$^+$ cells in close proximity to invading T cells in acute and chronic active human multiple sclerosis lesions further corroborates these findings.

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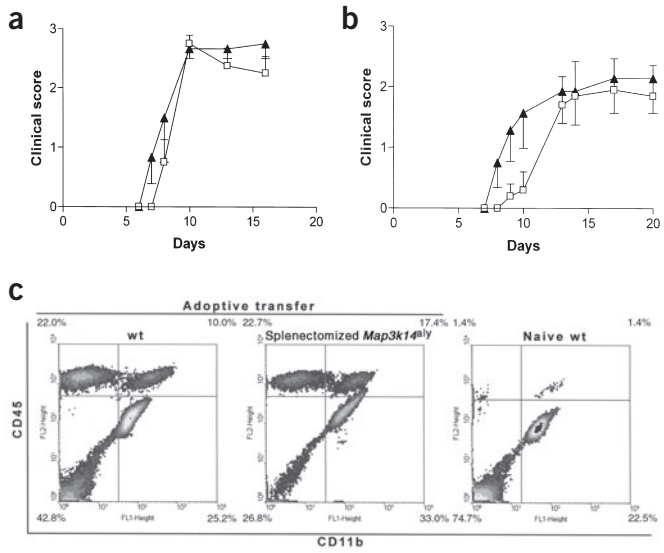


Figure 1 Secondary lymphoid tissues are not a pivotal homing site for encephalitogenic lymphocytes. (**a**) EAE was induced by adoptive transfer of MOG-reactive lymphocytes into *Map3k14*^{aly} (filled triangle) or wild-type (*Map3k14*$^{+/aly}$) mice (open square). (**b**) As in **a**, but using splenectomized *Map3k14*^{aly} mice (filled triangle) or wild-type mice (open square). No significant difference in the severity of the disease could be detected between the different groups. Shown is one representative of three experiments ($n = 6$ mice/group). (**c**) To quantify CNS infiltrates, CNS mononuclear cells from a group of four mice were isolated on day 20 after transfer as described and stained with antibodies to CD45 and CD11b and analyzed by flow cytometry. CD45 is used to distinguish CNS-resident microglia (CD45^{lo}) from invading leukocytes (CD45^{hi}). The CD45^{hi} cells are further distinguished by their expression of CD11b (macrophages and granulocytes) or lack thereof (lymphocytes). Naive C57BL/6 mice were used as a negative control.

RESULTS

Lymphocyte migration into the CNS is independent of lymphoid organs

It has been shown that upon adoptive transfer of encephalitogenic T cells into naive recipients, the T cells first migrate into secondary lymphoid tissues, where they evidently receive further maturation signals before CNS invasion[11]. To establish whether the lymphoreticular system is functionally involved in the guidance of committed autoreactive T cells into the CNS, we adoptively transferred myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide–reactive lymphocytes into *Map3k14*^{aly} (also known as *alymphoplasia* or *aly*) mice. *Map3k14*^{aly} mice, which carry a point mutation in the NF-κB-inducing kinase (NIK, encoded by *Map3k14*), are characterized by the complete absence of lymph nodes and Peyer's patches, and show a disorganized thymic and splenic architecture[13]. To further rule out splenic involvement in the T-cell homing process, we splenectomized *Map3k14*^{aly} mice before transferring MOG-reactive lymphocytes. *Map3k14*^{aly} mice (**Fig. 1a**) as well as splenectomized *Map3k14*^{aly} mice (**Fig. 1b**) are fully susceptible to adoptively transferred EAE when compared to wild-type control mice (**Fig. 1**). Using flow cytometry, we further show that the degree of CNS inflammation during peak disease is not diminished in the adoptively transferred, splenectomized *Map3k14*^{aly} mice compared to wild-type recipients (**Fig. 1c**). Before CNS infiltration, adoptively transferred lymphocytes seem to initially migrate into secondary lymphoid tissues (**Supplementary Fig. 1** online). But in the absence of a lymphoreticular system (splenectomized *Map3k14*^{aly}) adoptively transferred T cells can mainly be found in the blood before they invade the CNS (**Supplementary Fig. 1** online). Our data show that the migration of encephalitogenic T cells to secondary lymphoid tissues is not a prerequisite for the conditioning of auto-aggressive T cells before their migration into the CNS and the development of disease.

CNS-resident APCs do not present antigen *in vivo*

The above data implicate MHC class II–expressing APCs outside of secondary lymphoid sites as crucial for the development of EAE. With regard to the CNS, evidence has been presented that microglia[10,14,15] as well as astroglial cells[7] are capable of presenting antigen. Particularly, CNS-resident microglia have been shown to be potent APCs *in vitro* and are widely held to mediate target recognition of encephalitogenic T cells[14–17], although these findings have been challenged by others[9].

To dissect whether APCs in the CNS parenchyma or in the periphery present antigen to encephalitogenic T cells, we combined bone marrow chimerism with gene targeting as described earlier[18,19]. To track the reconstitution with bone marrow–derived elements we injected congenic bone marrow (C57BL/6-CD45.1) into lethally irradiated C57BL/6-CD45.2 recipient mice. Two months after reconstitution of wild-type mice with congenic bone marrow, cytofluorometric analysis of CNS mononucleated cells showed that microglia (CD45^{lo}/CD11b^{+}) were of host origin, whereas CD45^{hi} CNS-associated leukocytes were of donor origin (**Fig. 2a**). Detailed immunocytochemical analysis confirmed that CD45.1^{+} donor-derived cells were associated with blood vessels, but not the CNS parenchyma (**Fig. 2b**), consistent with reports by others[20,21].

In contrast to the radio-resistant CNS, the peripheral immune system is readily repopulated by cells derived from the donor bone marrow. MHC class II–bearing cells including B lymphocytes, DCs, monocytes, macrophages and polymorphonuclear cells within lymph nodes, spleen and blood are almost exclusively of bone marrow–donor genotype as assessed by flow cytometry and immunohistochemistry (**Supplementary Fig. 2** online).

To restrict capable APCs to either the CNS or peripheral immune compartment, we generated a combination of bone marrow chimeras using MHC class II–deficient (*H2-Ab1*$^{-/-}$, also known as *IA*β$^{b-/-}$) mice. Adoptive transfer of MOG-reactive lymphocytes into bone marrow chimeras with CNS-restricted MHC class II expression (*H2-Ab1*$^{-/-}$ → *H2-Ab1*$^{+/+}$) does not lead to CNS invasion and disease development (**Fig. 2c,d**). Notably, MHC class II expression restricted to the systemic immune compartment (*H2-Ab1*$^{+/+}$ → *H2-Ab1*$^{-/-}$) was permissive for disease development. The data indicate that CNS-resident APCs such as microglia or astrocytes are not required for T cells to recognize their cognate antigen *in vivo*. Even though secondary lymphoid tissues are not crucial for the migration of T cells into the CNS, reconstituting *H2-Ab1*$^{-/-}$ mice with *H2-Ab1*$^{+/+}$ bone marrow restored EAE susceptibility. We observed slight but significant differences between the susceptible groups ($P < 0.05$, days 13–17). The turnover of perivascular cells from the periphery into the CNS in bone marrow chimeric mice occurs over several weeks, as previously shown, whereas microglial replacement by fresh bone marrow emigrants is negligible[22]. Hence, the incomplete reconstitution of vessel-associated MHC class II–positive cells 6–8 weeks after bone marrow transfer readily explains the decrease in inflammation and clinical severity of bone marrow chimeras in which MHC class II molecules are absent from the CNS. Taken together, the data suggest that a radio-sensitive APC outside of the CNS parenchyma is obligatory for disease development.

MHC class II–positive DCs permit EAE development

Several recent reports claim that DCs not only migrate into the CNS during inflammation in multiple sclerosis lesions[23], but can also be found to associate with the blood-brain barrier and the meninges under

physiologic conditions[24–26]. To determine whether DCs are sufficient to support the reactivation of fully primed MOG-reactive encephalitogenic T cells *in vivo*, we used mice in which *H2-Ab1* expression was targeted specifically to the DCs of *H2-Ab1*$^{-/-}$ mice. Expression of *H2-Ab1* under the control of the *CD11c* promoter (hereafter transgenic mice referred to as *CD11c–H2-Ab1/H2-Ab1*$^{-/-}$) has been shown to be restricted to DCs[27,28]. **Fig. 3a**,**b** shows the distribution of MHC class II and CD11c in the spleen of *CD11c–H2-Ab1/H2-Ab1*$^{-/-}$ compared to *H2-Ab1*$^{+/+}$ and *H2-Ab1*$^{-/-}$ mice. As expected, we were not able to detect any expression of MHC class II molecules within the CNS parenchyma of *CD11c–H2-Ab1/H2-Ab1*$^{-/-}$ mice (**Fig. 3c**). MHC class II–positive cells, although small in number, were restricted to the meninges and vessels (**Fig. 3c**). Adoptive transfer of MOG-reactive lymphocytes into these mice showed that *CD11c*-driven *H2-Ab1* expression fully restores EAE susceptibility in *H2-Ab1*$^{-/-}$ mice (**Fig. 4a**). **Fig. 4b** shows the inflammatory lesions correlating with *H2-Ab1* expression. Taken together, CD11c^{+} DCs, even in the absence of other MHC class II–expressing cell types, are sufficient to permit disease development and CNS inflammation upon adoptive transfer of encephalitogenic T lymphocytes.

To determine whether an analogous population of CNS-associated DCs can also be found in human CNS tissue, we analyzed brain tissue obtained from multiple sclerosis patient autopsy material with confirmed diagnosis of acute or chronic active multiple sclerosis, as well as from noninflamed control tissue. As expected, within multiple sclerosis plaques we found a variety of immune cells including T cells (CD3, CD4), macrophages (CD68, CD163) (**Fig. 5**) and B cells/plasma cells (CD20, CD38, CD138) (data not shown). In addition, the inflamed tissue shows a drastic upregulation of human leukocyte antigen (HLA) class II molecules. In accordance with a previous study[23], we could also detect CD209 (DC-SIGN)$^{+}$ cells—a marker reported to be restricted to DCs[29]—in close proximity to invading T cells (**Fig. 5**). Notably, CD209^{+} cells were also found in noninflamed human brain tissue lining blood vessels, leading to the idea that they may act as the initial APCs recognized by invading encephalitogenic T cells.

Augmenting the number of DCs increases clinical severity

Given that DCs alone seem to be sufficient to allow T-cell target recognition and disease development during EAE, we determined

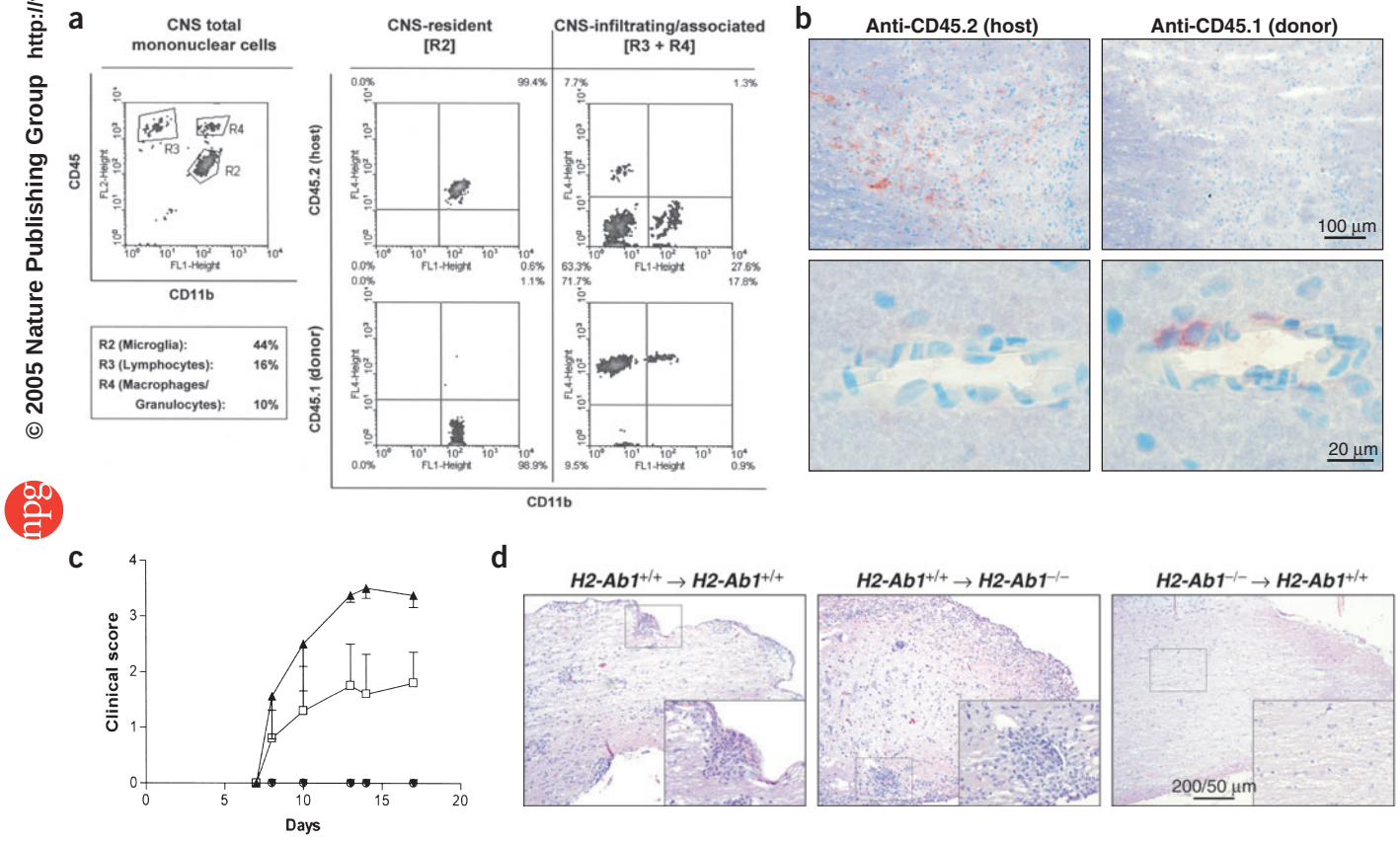


Figure 2 MHC class II molecules in the CNS parenchyma are not crucial for the development of EAE. Lethally irradiated C57BL/6-CD45.2 mice were reconstituted with bone marrow cells of C57BL/6-CD45.1 mice. Reconstitution of the CNS in bone marrow chimeras by donor-derived hematopoietic cells was determined by FACS analysis (**a**). Cells present in the spinal cords of C57BL/6-CD45.1 → C57BL/6-CD45.2 bone marrow chimeras were analyzed by flow cytometry. Cells were stained with antibodies to CD45 and CD11b (R2, CD45^{lo}/CD11b^{+} microglia; R3, CD45^{hi}/CD11b^{-} lymphocytes; R4, CD45^{hi}/CD11b^{+} macrophages and granulocytes). CNS-resident (R2) and associated cells (R3, R4) were further analyzed for the expression of the donor (CD45.1) versus host congenic marker (CD45.2) (right). (**b**) Frozen brain tissue of C57BL/6-CD45.1 → C57BL/6-CD45.2 was sectioned and stained for either the donor marker (CD45.1) or the host marker (CD45.2). Low magnification overviews (upper) and higher magnifications (lower). Donor-derived cells were restricted to blood vessels. (**c**) EAE was induced in bone marrow chimeras by adoptive transfer. Bone marrow chimeras lacking MHC class II molecules in the systemic immune compartment (*H2-Ab1*$^{-/-}$ → *H2-Ab1*$^{-/-}$, open circle; *H2-Ab1*$^{-/-}$ → *H2-Ab1*$^{+/+}$, filled inverted triangle) are resistant to the induction of EAE. Bone marrow chimeras expressing MHC class II molecules in the systemic immune compartment (*H2-Ab1*$^{+/+}$ → *H2-Ab1*$^{+/+}$, filled upright triangle; *H2-Ab1*$^{+/+}$ → *H2-Ab1*$^{-/-}$, open square) are susceptible to EAE. Shown is one representative of three experiments ($n \geq 6$ mice/ group). (**d**) Hematoxylin and eosin staining of spinal cord sections of bone marrow chimeras 10 d after disease onset. The tissue location of the higher magnification image is indicated by a rectangle.

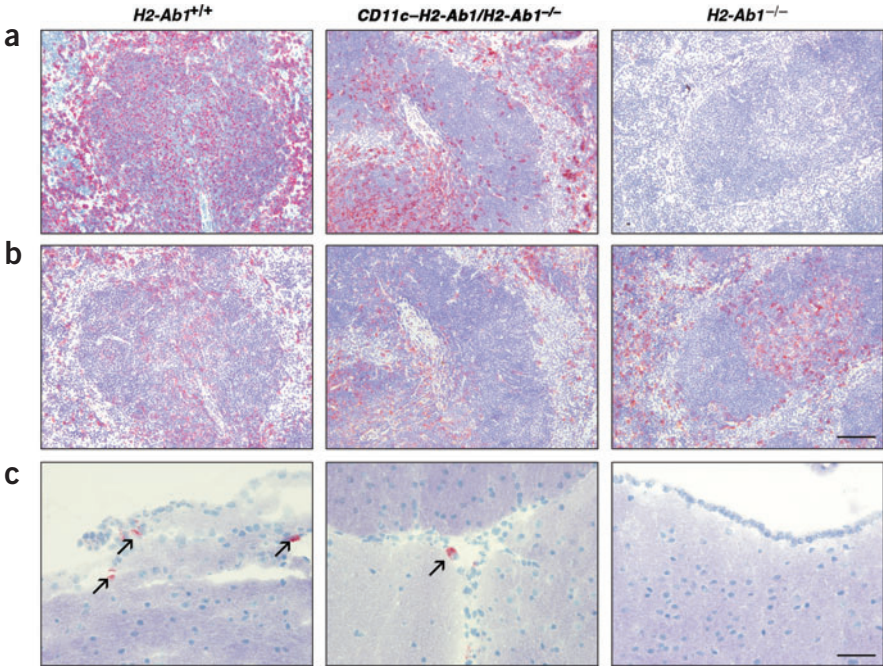
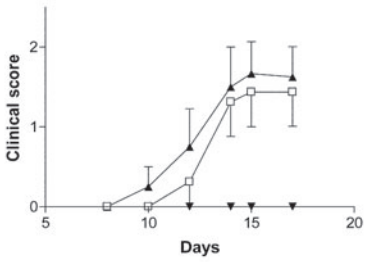


Figure 3 Localization of MHC class II–expressing cells in *CD11c–H2-Ab1/H2-Ab1*$^{-/-}$ mice. Spleen cryosections were prepared from *H2-Ab1*$^{+/+}$, *CD11c-H2-Ab1/H2-Ab1*$^{-/-}$ mice, and *H2-Ab1*$^{-/-}$ mice and stained with monoclonal antibodies against (**a**) MHC class II (M5/140), (**b**) CD11c. Staining shows a typical distribution of DCs in the spleen of *CD11c–H2-Ab1/H2-Ab1*$^{-/-}$ mice. Scale bar, 100 μm. (**c**) In brain sections, MHC class II can not be detected in the CNS parenchyma of *CD11c–H2-Ab1/H2-Ab1*$^{-/-}$ mice but on DCs located at the meninges or perivascular sites (arrows). Scale bar, 50 μm.

whether the limited number of suitable APCs is rate limiting and responsible for the lag phase between adoptive transfer of encephalitogenic T cells and CNS infiltration. We used recombinant FMS-like tyrosine kinase 3 ligand Flt-3L:IgG, a growth factor for DCs[30,31], to stimulate DC expansion. Upon systemic injection of (Flt-3L):IgG into recipient mice for 10 d, the number of DCs rose considerably in the lymphoreticular system. As for the CNS, we observed an increase of DCs in the meninges (**Fig. 6a** and **Supplementary Fig. 3** online). Detailed analysis shows that these DCs are predominantly CD11b^{+} myeloid DCs and have a mature phenotype as judged by the expression of CD80, CD86 and MHC class II (**Supplementary Fig. 3** online).

At day 5 of Flt-3L:IgG treatment, recipients were injected with MOG-reactive lymphocytes. Flt-3L:IgG-treated mice showed a substantial increase in disease severity (**Fig. 6b**). Lymphocyte infiltration was increased by ~30% as judged by the number of CD45^{hi} lymphocytes isolated from the CNS of Flt-3L:IgG-treated EAE mice (**Supplementary Fig. 3** online). To eliminate the potential role of Flt-3L:IgG as an adjuvant for bystander lymphocytes, we transferred encephalitogenic lymphocytes into Flt-3L:IgG-treated recombinase activating gene 1 (*Rag1*)-deficient mice (**Fig. 6c**), which lack mature lymphocytes, as well as to splenectomized *Map3k14*^{aly} mice (**Supplementary Fig. 3** online). Notably, Flt-3L:IgG treatment was found to condense the time span between adoptive transfer and disease onset and increased disease severity. In the inflamed brain tissue, T cells and DCs colocalize and Flt-3L:IgG treatment increased the size of the inflammatory lesion (**Fig. 6d**). These data corroborate our findings that DCs are the most, if not the only, critical population of APCs conferring CNS inflammation and disease development by encephalitogenic lymphocytes.



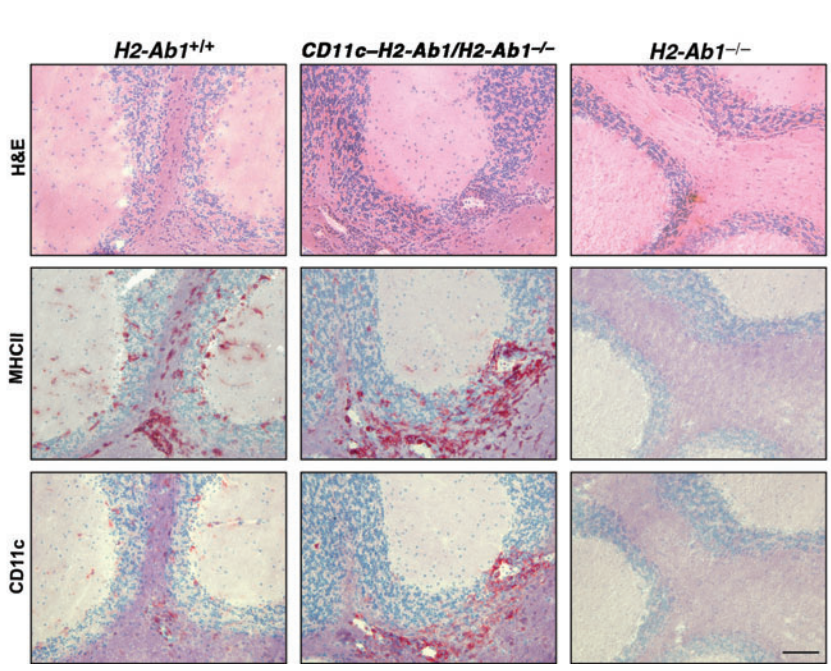


Figure 4 DC-restricted MHC class II expression is sufficient to permit disease development. MOG-reactive lymphocytes were injected into either *H2-Ab1*$^{+/+}$, *H2-Ab1*$^{-/-}$ or *CD11c–H2-Ab1/H2-Ab1*$^{-/-}$ mice. (**a**) Whereas *H2-Ab1*$^{-/-}$ mice did not develop clinical EAE (filled inverted triangle), *H2-Ab1*$^{+/+}$ (filled upright triangle) as well as *CD11c–H2-Ab1/H2-Ab1*$^{-/-}$ (open square) mice were equally susceptible to EAE. Shown is one representative of three experiments, $n \geq 6$ mice/group. (**b**) Ten days after disease onset, the mice were killed and CNS tissues were cryopreserved. Adjacent cerebellar sections were stained with monoclonal antibodies against MHC class II or CD11c and compared to hematoxylin and eosin–stained sections. Scale bar, 100 μm.

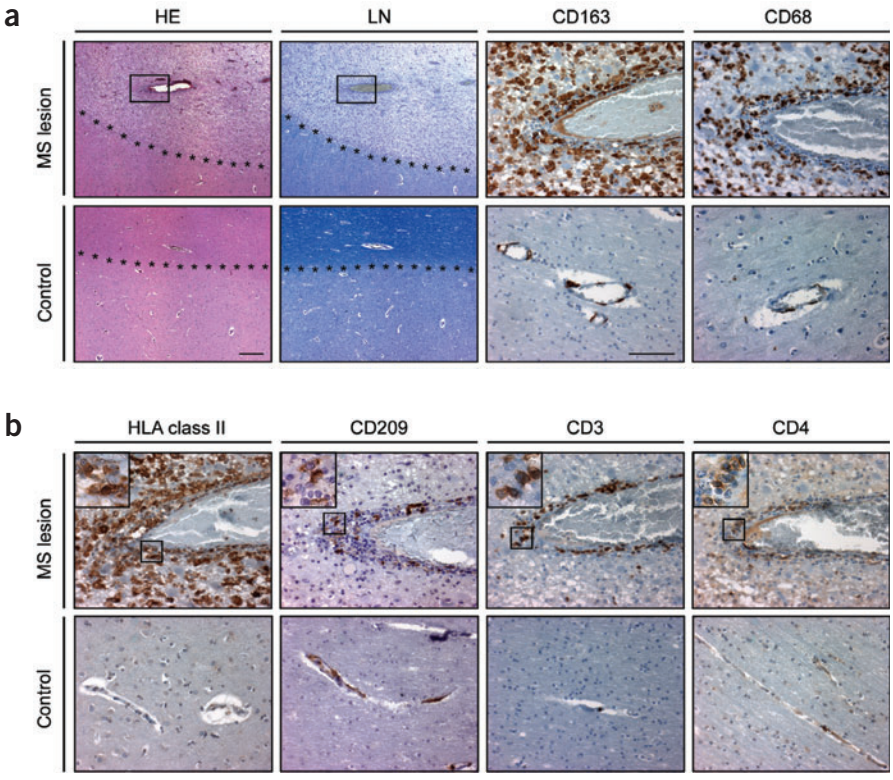


Figure 5 Presence of vessel-associated CD209+ cells in multiple sclerosis lesions (**a**) CNS tissue of multiple sclerosis patient autopsy material displaying a subcortical chronic active multiple sclerosis lesion characterized by severe demyelination (HE, hematoxylin and eosin; LN, Luxol-Nissl) and numerous CD163+ or CD68+ macrophage and microglia–rich inflammatory infiltrates including prominent perivascular inflammation (third and fourth upper panel). Control autopsy material (lower panels); no inflammatory infiltrates were observed and CD163 and CD68 show vessel-associated macrophages. Gray (bottom) and white matter (top) in first and second column is separated by asterisks. Rectangles represent the area chosen for higher-magnification analysis throughout. (**b**) Although strong HLA class II immunoreactivity was present throughout the multiple sclerosis lesion, CD209+ cells were vessel-associated and seen in close proximity to invading CD3+ and CD4+ T cells. In noninflamed tissue, CD209+ cells were found exclusively associated with blood vessels. Scale bar, 400 μm, first and second column in **a**; 100 μm for all other panels.

DISCUSSION

Given that oligodendrocytes are not capable of presenting antigen to encephalitogenic CD4+ T_H cells[4], an APC as a compulsory 'third party' is required for target recognition by encephalitogenic T cells to initiate EAE. There are three major sites at which adoptively transferred encephalitogenic lymphocytes can encounter competent APCs: (i) the systemic immune compartment with its secondary lymphoid tissues, (ii) the CNS parenchyma harboring microglia and astrocytes, (iii) the perivascular and meningeal space with its macrophages and DCs.

There is an enduring debate regarding the anatomic site responsible for the reactivation of encephalitogenic T cells and the development of disease. The importance of T-cell reactivation in the peripheral immune compartment is one issue, and the functional role of CNS APCs is another. With regard to the systemic immune compartment, the fate of encephalitogenic lymphocytes upon adoptive transfer into recipient animals remains unresolved. The normal homing behavior of transferred T cells seems to direct them into secondary lymphoid sites prior to CNS invasion[11]. It was shown that myelin-specific lymphocytes transferred into rats first enter secondary lymphoid tissues, where the expression of activation markers is synchronized before CNS entry[11]. In particular, myelin-specific lymphocytes entering secondary lymphoid tissues evidently mature within such a lymphoid environment as they upregulate chemokine receptors like CCR1, CCR3, CCR5 and CXCR4 (ref. 11). Our results confirm that in the presence of secondary lymphoid sites, encephalitogenic lymphocytes predominantly migrate into these organs, but this migration pattern is not essential to ultimately invade the CNS. We show that even in the complete absence of lymph nodes, Peyer's patches and spleen, encephalitogenic lymphocytes still find their way into the CNS and initiate tissue destruction with similar kinetics as seen in wild-type mice showing a normal lymphoreticular environment.

Within the CNS, astrocytes and endothelial cells, in principle, are capable of expressing MHC class II molecules, but their capacity to act as competent APCs remains speculative[6,32]. Although CNS-resident astrocytes have been suggested to potentially act as APCs for invading T cells[33], transgenic targeting of MHC class II expression to astrocytes did not support target recognition by encephalitogenic T cells *in vivo*[32]. Microglia and macrophages are generally considered the most prominent CNS-resident APCs and their capacity to present antigen *in vitro* has been repeatedly shown by several independent groups, including ours[15–17,34,35]. Furthermore, we and others have recently reported that lymphocyte-induced activation of microglia *in vivo* is crucial for immune infiltration of the CNS and the maintenance of encephalitogenicity during the effector phase of EAE[18,19,36,37]. To seek proof whether microglia are also capable of presenting antigen to auto-aggressive effector T cells *in vivo*, we restricted MHC class II expression either to the CNS parenchyma or to the peripheral immune compartment and the blood-brain barrier and meninges. Although microglia are vital components of the effector phase in EAE, the data presented here show that neither microglia, nor any other parenchymal elements are required to mediate cognate T cell–APC interactions *in vivo*. This conclusion can be drawn from the experiments using MHC class II bone marrow chimeras as well as using mice in which MHC class II expression was restricted to DCs.

Our data show that MHC class II expression restricted to DCs is permissive for full disease development induced by adoptive transfer of encephalitogenic lymphocytes. Taken together with the observation that neither secondary lymphoid tissues nor CNS-resident cells are required or sufficient to permit EAE development, we postulate that a population of DCs associated with the meninges and CNS blood vessels allows encephalitogenic T cells to recognize their cognate antigen leading to CNS invasion, inflammation and ultimately neurologic deficit. Although DCs seem to be completely absent from the CNS parenchyma under physiological, nonpathological conditions, several groups showed the presence of DCs in the meninges and choroid plexus of rodents[7,23–25]. We confirmed the presence of DCs at the blood-brain barrier and meninges in mice, and analogous CD209+ cells were found in noninflamed human brain tissue, suggesting that initial antigen presentation could potentially occur at the blood-brain barrier, thus promoting entry of T cells into the CNS[25,38,39]. Under physiologic conditions, DCs associated with CNS vessels could pro-

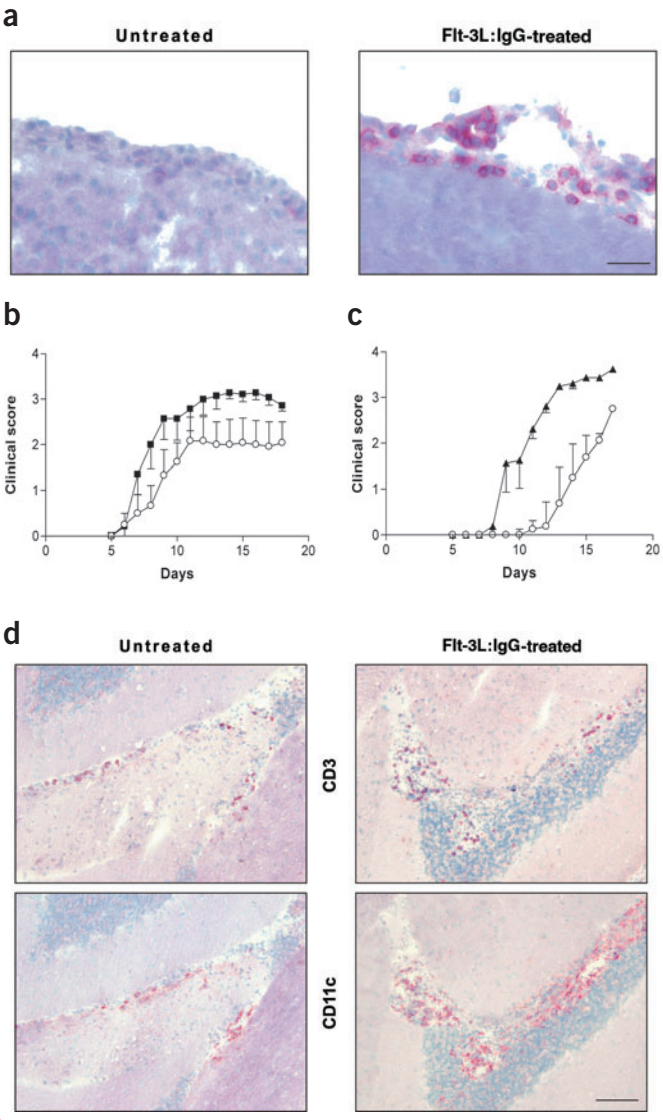


Figure 6 Augmenting the number of DCs increases EAE severity. (**a**) C57BL/6 mice were treated with (Flt-3L):IgG as described[29]. Cryosections of brains were stained using monoclonal antibodies against CD11c, showing a strong increase of immunoreactivity in the meninges of (Flt-3L):IgG-treated mice. Scale bar, 50 μm. (**b**) C57BL/6 mice or (**c**) *Rag1*$^{-/-}$ mice were treated with (Flt-3L):IgG for 5 d before adoptive transfer of MOG-primed lymphocytes. Treatment with Flt-3L:IgG was continued for an additional 5d. (**b**,**c**) Mice treated with (Flt-3L):IgG (filled square or triangle) develop significantly more severe EAE than mock-treated control mice (open circle). In **b**, $P < 0.05$, days 10–20. In **c**, $P < 0.05$, days 8–17. Shown is a representative of three individual experiments ($n \geq 6$ mice/group). (**d**) Mice were killed and CNS tissues were cryopreserved 10 d after disease onset. Staining for CD3 and CD11c shows that inflammatory T cells colocalize with DCs in the inflamed brain. Scale bar, 100 μm.

cess and present myelin antigens in the context of MHC class II molecules. Their maturation state under physiologic conditions may not be sufficient to drive naive T cells, but would be adequate to restimulate adoptively transferred antigen-experienced effector cells to recognize their cognate antigen. Alternatively, under pathological conditions, it is feasible that DCs migrate into the CNS in order to act as APCs for comigrating T cells. Inflammatory foci in the CNS arrange in a fashion similar to secondary lymphoid organs[38,39], and other studies indicate that epitope spreading occurs exclusively within the inflamed CNS[40], suggesting that the CNS could act as a neolymphoid tissue. To underline the vital role of DCs to provide target recognition cues for committed encephalitogenic T cells, we could show that increasing the number of available DCs substantially increases the severity of clinical symptoms. It is widely held that like EAE, the pathogenesis of multiple sclerosis is mainly driven by CNS-invading encephalitogenic T_H cells. Although EAE is an inducible rather than spontaneous model for multiple sclerosis, clearly limiting its translatability to the human disease, fundamental mechanisms, such as antigen recognition, are not likely to differ considerably between humans and mice. We propose that DCs, strategically located between the immune system and CNS, act as sentinels for the CNS, scanning for physiologic self or infectious foreign antigens and, as part of the normal immune surveillance and protection of the CNS, present these to cognate T cells migrating through CNS vessels. Thus, DCs at the interface of the CNS and the immune system are pivotal players in the development of CNS inflammation and could serve as a novel therapeutic target for the treatment of patients with multiple sclerosis and other inflammatory diseases of the CNS.

METHODS

Mice. C57BL/6 and C57BL/6-CD45.1 mice were purchased from Harlan Laboratories. The following mice were bred in house under specific pathogen-free conditions: *Rag1*$^{-/-}$, *CD11c–H2-Ab1/H2-Ab1*$^{-/-}$, *H2-Ab1*$^{-/-}$, *Map3k14*^{aly}. Heterozygous *Map3k14*$^{+/aly}$ mice were used as a control for *Map3k14*^{aly} mice. Bone marrow chimeras were generated as described previously[18,19]. After recovery, bone marrow chimeras were bled retro-orbitally to ensure more than 95% engraftment of blood leukocytes. All experiments involving animals were approved by the Swiss Kantonal Veterinary office (2003/68).

Flt-3L:IgG treatment of mice. Flt-3L:IgG was purchased from BioExpress. Mice were treated with Flt-3L:IgG (10 μg/mouse/d) for 10 d consecutively. In adoptive-transfer EAE experiments, mice were treated with Flt-3L:IgG for 5 d before adoptive transfer. Flt-3L:IgG treatment was continued for an additional 5 d.

Splenectomy. We anesthetized mice using an isoflurane inhalator. A 0.5-cm skin incision was made at the level of the thirteenth rib, the spleen was exposed by opening the abdominal wall and exteriorized through the incision. We ligated blood vessels using four nylon sutures and removed the spleen. The peritoneal wall and the skin incision were closed with Indermil tissue adhesive glue (Tyco Healthcare). We treated mice with 5 μg/g body weight flunixin subcutaneously for 2 d and 0.75% Borgal in the drinking water for 7 d after surgery.

Induction of EAE. We obtained $MOG_{35–55}$ peptide (amino acid sequence: MEVGWYRSPFSRVVHLYRNGK) from Research Genetics. $MOG_{35–55}$-reactive lymphocytes were generated as described[18]. Cells were injected into recipient mice ($20–30 \times 10^6$ cells/mouse). We administered pertussis toxin to mice (200 ng/mouse) on day 0 and 2 ng/mouse after transfer. Mice were scored as follows: 0, no detectable signs of EAE; 0.5, distal limp tail; 1, complete limp tail; 1.5, limp tail and hind limb weakness; 2, unilateral partial hind limb paralysis; 2.5, bilateral partial hind limb paralysis; 3, complete bilateral hind limb paralysis; 3.5, complete hind limb paralysis and unilateral forelimb paralysis; 4, total paralysis of fore and hind limbs (score > 4, to be killed); 5, death. Each time point shown is the average disease score of each group ± s.e.m. Statistical significance was assessed using a unpaired Student *t*-Test.

Histology. Whole mouse brains or spinal columns were fixed in 4% paraformaldehyde in phosphate-buffered saline and paraffin-embedded or freshly snap-frozen in liquid nitrogen according to standard protocols. We used the following mouse-specific antibodies: CD11c (Jackson ImmunoResearch Labs), MHC class II (M5/114, American Type Culture Collection) CD3, CD45.1, CD45.2 (BD Pharmingen). Primary antibodies were shown by sequential incubation with goat antibodies against species-specific immunoglobulins, followed by alkaline phosphatase–labeled donkey antibodies against goat immunoglobulins (Jackson ImmunoResearch). We visualized alkaline phosphatase using naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid-2-methoxy anilide) phosphate and new fuchsin as substrate. Endogenous alkaline phosphatase was blocked by levamisole.

Color reactions were performed at room temperature (21–23 °C) for 15 min with reagents from Sigma Chemical Co. We counterstained sections with hemalum and mounted coverslips with glycerol and gelatin.

Formalin-fixed and paraffin-embedded human autopsy material of acute, chronic active and chronic inactive multiple sclerosis cases as well as age-matched controls without neuropathological alteration was used. Informed consent for autopsy was given by the relatives. All cases underwent detailed neuropathologic examination. We performed hematoxylin and eosin and Luxol-Nissl stains according to standard procedures. Immunohistochemical stains were carried out on an automated Nexus staining apparatus (Ventana Medical Systems), following the manufacturer's guidelines. We used antibodies to human CD8, CD20, CD68, CD79a, CD138 (all DAKO), CD4, CD38, CD163, HLA DR,DP,DQ (MHC class II; all Novocastra Laboratories Ltd.), CD3 (Labvision) and CD209 (R&D Systems).

Flow cytometry. For cytofluorometric and immunohistochemical analysis, we used the following antibodies: CD45-pan, CD45.1, CD45.2, CD11b, CD11c, CD80, CD86, H2-Ab1. All antibodies were obtained from BD Pharmingen. Cytofluorometric analysis of CNS invading lymphocytes has been described previously[18]. Briefly, mice were killed with CO_2 and spinal cords were removed by flushing the spinal column with sterile HBSS. We dissected the brain to isolate the brain stem. Both tissues were homogenized and strained through a 100 µm nylon filter (Fisher). After centrifugation, we resuspended the cell suspension in 37% isotonic Percoll and underlayed it with 70% isotonic Percoll. The gradient was centrifuged at 600 g for 25 min at room temperature (21–23 °C). We collected the interphase cells and extensively washed them before staining. For flow cytometry, the cells were stained with primary antibodies (all from BD Pharmingen) for 30 min at 4 °C, washed and, when required, incubated with streptavidin-coupled fluorochromes (BD Pharmingen) for 15 min. We washed and analyzed the cells using a FACSCalibur (BD Pharmingen) with CellQuest software. Post-acquisition analysis was performed using WinMDI 2.8 software (Scripps-Research Institute).

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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DISCUSSION

The sequence of immunopathological events involved in the development of EAE can be subdivided into two phases: i) T cell priming/activation, which is initiated by systemic immunization with myelin Ags and ii) the subsequent recruitment/effector phase, in which primed myelin-reactive T cells reencounter their cognate Ag in the target tissue leading to the development of EAE. In this thesis, I have analyzed the role of SLTs and their requirement for the "T cell priming phase" and determined the cell types, which are necessary and sufficient during the "effector phase" to initiate an inflammatory cascade leading to the tissue lesion.

T CELL PRIMING PHASE

T CELL PRIMING IN THE ABSENCE OF SLTS

A fundamental tenet of immunology is the notion that adaptive immune responses are generated in SLTs (23). SLTs contain defined B and T cell zones which provide the structural requirements for the concerted interaction of immune cells. Nearly 40 years ago it was shown that the presence of lymphatic vessels and LNs is a requirement for the development of an immune response. Isolated skin flaps were connected to recipient guinea pigs. An immune response to the skin-derived Ag was only provoked when the skin flaps were connected to the recipients via lymphatic vessels and in the presence of LNs (138). Many studies using mutant mice strains have further highlighted the pivotal role of these specialized tissues. $LT\alpha^{-/-}$ mice, which lack all LNs, can not mount Ab responses and class switching upon immunization with sheep red blood cells (139). *Aly/aly* mice, which are also characterized by the absence of all LNs and Peyer's patches, are highly susceptible to VSV infections caused by the failure to mount IgG responses (43). The treatment of wt mice with LTβR-Fc protein disrupts B cell follicles in SLTs. As a result of the destroyed structures, humoral immune responses are suppressed (140). These reports verify that GCs provide a vital milieu for the generation of high-affinity Abs and isotype switching. No such dedicated structures however are evident for T cell responses. Recent methods using intravital microscopy have shed new light on T cell/DC communications within LNs

(141). The complex motility behavior of T cells is believed to comprise different stages and serial interactions with DCs in SLTs. Nevertheless, only few studies provide insight into the SLT-requirement for effective T cell priming. $LT\alpha^{-/-}$ mice do not elicit a contact-hypersensitivity reaction in response to epicutaneous hapten indicating that LNs comprise a necessity for T cell-mediated immune responses (142). It is widely held that immune responses to an allograft are prompted by the actions of both $CD4^+$ and $CD8^+$ T cells. However, it has been a subject of debate whether T cell-mediated immune responses to an allograft are initiated in the allograft itself or in SLTs. Several studies speak for the latter hypothesis. *Aly/aly* and splenectomized *aly/aly* mice are not able to reject allogeneic skin grafts or cardiac allografts indicating that the presence of lymphoid tissues is required for the development of an allograft response (143,24). Collectively, these studies postulate that expansion of Ag-specific T cells occurs within SLTs. This notion has also been applied to immunization-induced T cell priming.

We provide irrefutable evidence that in mice lacking SLTs, s.c. immunization with foreign as well as auto-Ag can initiate productive T cell priming as well as autoimmunity respectively. We could demonstrate that *aly/aly* mice mount solid delayed-type hypersensitivity (DTH) reactions, which is indicative of an effective T cell-mediated immune response. We further observed that *aly/aly* mice are resistant to EAE. However, we discovered that this cannot be explained by the developmental malformations of *aly/aly* mice. By generating BM-chimeric mice it was found that the NIK mutation abrogates the polarization of IL-17 producing encephalitogenic T cells even when the lymphoreticular compartment is unperturbed (NIK-deficient immune system and normal SLTs). So far the immunodeficiency observed in *aly/aly* mice was mainly attributed to the absence of LNs but not on the impact of NIK signaling on immunity (24,40). We could demonstrate that the immunodeficiency of *aly/aly* mice is disconnected from their structural malformations but due to an intrinsic defect in cells of the hematopoietic compartment.

Surprisingly, *aly* BM-chimeric mice, which lack all SLTs but harbour a normal immune system, are fully susceptible to EAE induced by s.c. immunization with MOG. This implies that even in the absence of SLTs, effective T cell priming can be initiated. In contrast to the potent T cell priming, humoral immune responses were

completely abrogated in these BM-chimeric mice, which was revealed by the absence of Ag-specific Abs.

Taken together, we have challenged the dogma that the initiation of a T cell-mediated adaptive immune response only functions in the presence of the structural environment of SLTs. As assessed by other groups, for B cell activation, high-affinity Abs and class switching, highly organized SLTs are obligatory. In contrast, T cell priming is structure-independent. Interestingly, this phenomenon can also be found in evolution. B and T cell receptors evolved simultaneously with the emergence of RAG in jawed vertebrates. While jawed fish exert adaptive immune functions, GC formation and Ig-class switching only evolved in amphibians/birds concomitant with the development of LNs. We have therefore recreated the immune-evolutionary stage of cold-blooded vertebrates in mice to prove that modern B cells are dependant on the topography of lymphoid tissues while T cells retain the capacity to recognize Ag in a structure-independent fashion.

ALTERNATIVE SITES FOR T CELL PRIMING

The question whether adaptive immune responses can be initiated in LN-deficient mice was investigated by several groups. In a study where LTα$^{-/-}$ mice were used, CD8^{+} T cell and B cell immunity to virus was elicited, the response was however delayed (144). Consistent with this result, we have also noted a delay in the proliferation of Ag-specific T cells in *aly/aly* mice. These findings were attributed to the lack of LNs (in *aly/aly* and LTα$^{-/-}$) but did not exclude the spleen as a compensatory organ for T cell priming. Fu *et al*. have demonstrated that LTα$^{-/-}$ and splenectomized LTβR$^{-/-}$ mice (lack all SLTs) were able to reject allogeneic skin grafts and cardiac allografts albeit with delayed kinetics in comparison to wt mice. They concluded that LNs are critical for the rejection of allografts but at the same time dispensable (145,146). Whether the allograft itself provides a T cell priming site remains to be shown. The discrepancy between LTα$^{-/-}$/LTβR$^{-/-}$ mice and *aly/aly* mice, which were not able to reject allogeneic grafts (41), is in agreement with our results. We assessed that the immunodeficiency seen in *aly/aly* mice can not be explained solely based on the absence of LNs and the structural alteration in the spleen.

Recently Mori *et al*. have analyzed whether the altered homing behavior of T cells into SLTs has an effect on the development of T cell immune responses (147). Paucity of LN T cell (*plt*) mice, which lack CCL21 and CCL19, display T cell migration defects into LNs. Despite the abrogated T cell zone, splenectomized *plt* mice exhibit delayed but effective T cell priming. This indicates that T cell priming can occur outside of dedicated T cell zones.

The question at which alternative site T cell priming could arise in mice lacking all SLTs was addressed by Schirrmacher *et al*. They demonstrated that BM as a primary lymphoid organ provides a potential site for T cell priming. Naïve ovalbumin-TCR transgenic $CD4^+$ (OTII) and $CD8^+$ (OTI) T cells were transferred into either wt or splenectomized *aly/aly* mice. The cells were found to home to BM (or spleen and LNs if present) where they were fully primed by resident DCs in response to blood-borne Ag. It was further observed that OTI cells primed in the BM of splenectomized *aly/aly* mice can elicit protective anti-tumor immunity. The fact that BM facilitates T cell priming to blood-borne Ag highlights again the dispensability of conventional SLTs.

Upon respiratory infections or inflammation in the lung, the generation of inducible bronchial associated lymphoid tissues (iBALT) can be observed. It was reported that such a rapid formation of iBALTs could be induced in splenectomized $LT\alpha^{-/-}$ mice upon intranasal injections of influenza virus (31). These iBALTs contained defined T and B cell areas which conducted effective T and B cell priming leading to clearance of the virus. Together, these reports emphasize our discovery that adaptive immunity can occur outside of dedicated lymphoid structures. We showed for the first time the induction of successful T cell priming and autoimmunity upon s.c. immunization in mice lacking SLTs. We demonstrated that the liver could potentially serve as a site for the development of T cell-mediated immune responses in the absence of SLTs. By using fluorescently coupled microspheres, it was shown that the Ag is carried by local APCs from the site of immunization into the liver when SLTs are absent. In the liver, the accumulation of Ag-specific TCR transgenic T cells was detected and endogenous IFNγ secreting cells specific for the Ag used in the immunization. In mammals, early fetal hematopoiesis takes place in the liver. It is possible that the adult liver is reminiscent of this function and can substitute regular SLTs when they are not available.

LYMPHOID NEOGENESIS

Tissues affected in autoimmune diseases such as MS, rheumatic arthritis and Hashimoto thyroiditis often contain TLTs (26). These ectopic lymphoid aggregates comprise distinct B cell compartments including GCs with FDCs and T cell compartments similar to the micro-architecture in SLTs. In MS, such lymphoid-like structures were identified in the meninges containing B cells, T cells and FDCs (148).

Aloisi *et al*. investigated whether such TLTs are involved in the progression and maintenance of EAE. EAE-diseased mice were treated with LTβR-Fc protein, which is known to disrupt lymphoid structures (140). They demonstrated that the LTβR-Fc treatment prevents relapses, T cell infiltration and the development of meningeal lymphoid follicles (149). It remains to be shown whether this observation is solely mediated by the disrupted LTβR pathway or due to an additional inhibition of LIGHT (150), which also binds LTβR and is implicated in T cell responses.

All our data pointed towards the liver as an alternative priming site in mice lacking SLTs. We discovered such lymphoid-like structures in the livers of SLT-deficient mice upon s.c. immunization with auto- or foreign Ag. Thus in the absence of SLTs, TLTs could substitute conventional SLTs and induce T cell priming locally. The ectopic structures in the livers contained mainly T cells and DCs and in contrast to other reports, only few FDCs and GCs. The fact that no GCs were detected in these ectopic lymphoid structures provides an explanation for the absence of Ag-specific Abs and proves that humoral immune responses are structure-dependant.

In order to define whether such newly formed TLTs can serve to propagate T cell expansion, we treated wt mice with LTβR-Fc protein. Although the formation of Abs and class-switching was eliminated, LTβR-Fc treatment did not influence the disease course of EAE. Similar findings were obtained by Gommerman *et al.* who showed that in EAE models requiring pertussis toxin, LT pathway inhibition upon LTBR-Fc treatment did not affect EAE development (151). This clearly implies that T cell-mediated immunity functions structure-independently while B-cell immunity requires a proper structural environment.

RECRUITMENT AND EFFECTOR PHASE

IDENTIFICATION OF CELL TYPES NECESSARY TO PERMIT IMMUNE INVASION OF THE CNS AND DISEASE DEVELOPMENT

Encephalitogenic $CD4^+$ T_H cells need to reencounter their cognate Ag in the context of MHCII molecules in the target tissue in order to initiate EAE. There are 3 major sites at which competent APCs could potentially present myelin-epitopes to encephalitogenic T cells: i) the systemic immune compartment with its SLTs, ii) the CNS parenchyma harboring microglia and iii) the perivascular and meningeal space with its CNS-associated DCs.

APCS RESIDING IN SLTS

Upon adoptive transfer of MOG-reactive T cells into recipient mice a lag period of several days can be observed prior to CNS invasion. Is was shown that myelin-reactive lymphocytes adoptively transferred into rats, they first migrate to SLTs where the expression of activation markers and chemokine receptors is synchronized before CNS entry (152). Cervical LNs were proposed to be crucial for the development of an immune response to brain-derived Ag (87,153). Their important role became further evident by the fact that cervical lymphadenectomy in Lewis rats strikingly attenuated cryolesion-induced EAE (154). It was demonstrated that upon i.c injection of Ag-loaded DCs, their migration from the CNS into the cervical LNs is both necessary and sufficient to activate Ag-specific T cells in order to promote their migration into the brain (90). When MBP-reactive T cells were injected intrathecally into Lewis rats, they first leave the CNS, become reactivated in the periphery and then invade the CNS (134). In summary, these studies indicate that encephalitogenic T cells home to (cervical) LNs where they encounter their cognate Ag prior to CNS invasion.

We clearly prove that mice lacking SLTs are fully susceptible to EAE induced by either adoptive transfer encephalitogenic T cells or by active s.c. immunization with MOG. Therefore it is evident that SLTs are dispensable for i) the priming of MOG-reactive T cells and ii) their reactivation preceding CNS invasion. This further

indicates that the presence of CNS-Ag as well as CNS-associated APCs in the cervical LNs is a result of CNS inflammation but not a driving force.

MICROGLIA – RESIDENT GUARDS OF THE CNS

Parenchymal microglial cells express all the appropriate restricting elements and co-stimulatory factors needed to elicit and support T cell responses (155,156,157). In many CNS diseases, parenchymal microglial cells are rapidly activated and upregulate MHCII, suggesting that they may participate in Ag presentation (158,159,160). There is no doubt that within the CNS parenchyma, microglial cells are fully equipped to act as APCs. Whether they mediate target recognition of encephalitogenic T cells remained to be proven. The observation that microglia are resistant to radiation while CNS-associated cells (DCs and macrophages) are radiation sensitive has allowed us to dissect the roles of CNS-endogenous versus CNS-associated cells in Ag-presentation to T cells in an *in vivo* setting. By combining BM-chimerism and gene-targeting, we restricted the APC-capability to either the CNS-parenchyma (microglia) or the systemic immune compartment. Upon transfer of MOG-reactive T cells, chimeras with MHCII expression restricted to the systemic immune compartment and CNS-associated cells were susceptible to EAE. However, mice with MHCII expression restricted to the CNS-parenchyma were resistant to the development of disease. These findings corroborate that cells within the CNS-parenchyma such as microglia or astrocytes do not serve as APCs permitting T cell entry.

Earlier reports stated that the contribution of microglial cells is vital for the development of EAE. In mice in which microglial cells were deficient of the costimulatory molecule CD40, infiltration of T cells was impaired and EAE severity diminished (117). Furthermore IL-23 secreted by CNS-resident cells is critical for the effector phase of EAE (161). In our studies, transgenic mice were used which allowed an inducible and transient inhibition of microglia. We could show that EAE was considerably repressed when microglial cells were transiently "paralysed". The inhibited activation of microglia led to a reduced inflammation of the CNS. This indicates that the release of reactive oxygen species intermediates in addition to cytokines and chemokines by activated microglia is vital for the development and

maintenance of EAE. Collectively, these observations reveal that while micoglia are crucial during the effector phase for the progression of EAE, they are not required to participate in Ag-presentation.

CNS-ASSOCIATED DCS – SENTINELS AT THE GATEWAY TO THE CNS

Under physiological conditions, DCs in the CNS reside in the meninges and the choroid plexus but not in the cerebellar parenchyma, brain stem or spinal cord (127) (125). Due to the strategically important location, the outermost basal lamina of the vessel wall endothelium and the one on top of the glia limitans CNS parenchyma, perivascular CNS-associated APCs are ideally situated to present Ag to invading myelin-reactive T cells. In the pathogenesis of EAE, during pre-disease onset, DCs can be found in the spinal cord white matter and during acute phase and relapse, DCs are present in the inflammatory CNS infiltrates (126) (125). These data suggest that intracerebral recruitment of DCs plays an important role for the onset and progression of the disease. So far it has remained unknown whether the DCs detected in the lesions during EAE are BM-derived or stem from DC-precursors located in the meninges. Some groups claim that microglial cells resemble myeloid precursors in that they display the capacity to differentiate into DCs depending on the cytokine environment. It was shown that $CD11c^+$ cells can be generated *ex vivo* from microglial cells upon GM-CSF stimulation (162,163). However, while these DCs in the inflamed CNS exhibited a mature phenotype and induced T cell proliferation, no *in vivo* evidence was provided that microglial cells differentiate into DCs. By using BM-chimeras with congenic markers, we assessed that $CD11c^+$ cells isolated from the CNS were exclusively BM-derived and not of parenchymal origin (microglia).

Whether CNS-associated DCs participate in Ag-presentation and CNS invasion during EAE has so far not been established. Almost 20 years ago, Hickey and Kimura demonstrated that blood-borne “perivascular microglial cells” (CNS-associated APCs) were competent candidates to present Ag to infiltrating T cells (132). Encephalitogenic T cells were adoptively transferred into BM-chimeric rats, in which MHCII recognition was restricted to BM-derived cells. They demonstrated that hematopoietic derived APCs were necessary and sufficient to confer disease. This seminal work was definitely the first claiming that perivascular located APCs could

play a major role in mediating CNS-inflammation. It was however not addressed whether the encephalitogenic T cells were reactivated in SLTs prior to CNS-invasion. The capacity of other hematopoietic-derived cells such as B cells or macrophages to function as competent APCs was not excluded in their model.

Using transgenic mice in which MHCII is exclusively targeted to DCs (all other potential APCs including microglia were MHCII-deficient), we showed that DCs alone are sufficient to present the cognate Ag to MOG-reactive T cell and are permissive for the development of CNS-inflammation. Furthermore, by augmenting the number of CNS-associated DCs, upon adoptive transfer with MOG-reactive T cells, the disease severity of EAE is increased and the onset of the disease is earlier. Detailed analysis of CNS-DCs during EAE revealed that they were predominantly myeloid ($CD11b^+$), of a mature phenotype and were exclusively BM-derived. In MS lesions, analogous cells, $CD209^+$ DCs, were detected associated with vessels and in close proximity to invading T cells. These data indicate that DCs at the interface of the immune system and the CNS are the crucial APCs conferring CNS inflammation of encephalitogenic T cells.

Epitope spreading has been well described in inflammatory diseases such as MS, in relapsing-remitting EAE and TMEV-IDD (108). It is a process in which reactivity to a different epitope than the disease-inducing epitope occurs. In agreement with our results, McMahon *et al.* demonstrated in two mouse models of EAE, that epitope spreading occurs directly in the CNS and not in peripheral lymphoid tissues (164). They showed that DCs were the most critical APC population associated with the CNS to initiate epitope spreading in the inflamed CNS. Neither microglia nor macrophages isolated form the CNS were capable of stimulating naive myelin-specific T cells. Collectively, these *in vivo* studies strongly suggest that CNS-associated DCs are able to initiate both primary and secondary T cell responses locally. Miller *et al*. further determined the DC subset in the inflamed CNS that drives epitope spreading (165). Myeloid DCs, in comparison to lymphoid DCs, plasmacytoid DCs and macrophages, were the most efficient in presenting endogenous peptide as assessed by the IL-17 production of $CD4^+$ T cells. Consistent with our results, they also observed that these DCs originated in the BM.

Taken together, CNS-associated and not CNS resident cells permit the recognition of the cognate neuroantigen by CNS invading T cells. However, CNS-resident microglia are key players for the maintenance of encephalitogenicity during an immune response.

In MS, neuroantigen-specific T cells, which have escaped tolerance, invade the CNS where they initiate a cascade leading to tissue damage. It is definitely established that DCs are not only decisive for T cell priming but also T cell tolerance. Several groups investigated whether Ags from the CNS could be utilized to induce peripheral tolerance and hence prevent the development of the disease. Injections of DCs matured with TNF-α and pulsed with MOG were shown to ameliorate the EAE disease course (166). Moreover, injection of DCs pulsed with MBP induced tolerance and prevented EAE in Lewis rats (167). Interestingly, Suter *et al*. identified CNS-localized DCs in the inflamed CNS with regulatory properties (168). DCs that were isolated from the CNS of EAE-diseased mice were immature as defined by the intermediate and low expression of MHCII and CD80 respectively. These DCs were not capable to induce priming of naïve T cells, on the contrary, T cell proliferation was inhibited. The presence of such regulatory DCs might contribute to the "immune privilege" of the CNS and the limitation of infiltration and disease progression.

CONCLUDING REMARKS

In this thesis, I have discovered that immunization-induced expansion of T lymphocytes can occur outside of SLTs. While B cell maturation including the production of Abs is highly dependent on the topography of SLTs, T cell immunity can develop completely independent of such dedicated lymphoid tissues. The finding that SLTs are not a prerequisite for T cell priming has major implications for our understanding of the development and evolution of adaptive immune responses and is of great interest in the context of vaccination. While intra-nodal immunization has been considered as an effective route of vaccination, our findings suggest that this concept needs to be revised. As for the development of autoimmune diseases, self-reactive T cells may not need to be primed in tissue-draining LNs, but could occur at the inflammatory site or even in organs distant to the target tissue. Our results point towards the liver as an alternative site for successful T cell priming independent of the topography of dedicated lymphoid tissues. However, it is feasible that other organs, such as the CNS, provide an environment in which auto-aggressive T cells can be generated.

We could demonstrate that when CNS-myelin is the target for such self-reactive T lymphocytes, DCs in perivascular spaces are sufficient to present the cognate Ag to these T cells in order to mediate CNS-inflammation. After target recognition, self-reactive T cells migrate through the vessels into the CNS parenchyma where they interact with microglia. We have established that microglia are not crucial for T cell target recognition but are an absolute requirement for the progression of the inflammatory cascade leading to the development of EAE. While the precise factors that lead to the collateral tissue damage of myelinating tissue remain to be definitively elucidated, our current knowledge on target recognition and the location of vital T cell-APC interactions could lead to the development of novel therapeutic strategies for the treatment of inflammatory CNS diseases. Under physiological conditions, CNS-associated DCs play a role in mediating immune surveillance of the CNS. In CNS-mediated autoimmune diseases, a therapeutic strategy could be to target these DCs at the interface of the immune system and the CNS in order to reestablish peripheral immune tolerance against self and inhibit the progression of T lymphocytes into the neuropil.

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